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THE TOXICITY OF SULPHADIAZINE AND SULPHAGUANIDINE TO MACROPHAGES *IN VITRO*.

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LOCAL application of sulphonamides to wounds as a prophylactic or therapeutic measure is now widely favoured and has become, if not more, at least a useful adjunct to formal surgery. An earlier study (Jacoby, Medawar and Willmer, 1941) of the effects of some of the sulphonamides on tissue cells *in vitro* was, therefore, extended to two further compounds of this group, viz. sulphadiazine and sulphaguanidine. The use of both these drugs in clinical and surgical therapy is of a more recent date, and various claims have been made for their particular usefulness in preference to some of the older members of this series of drugs. Sulphadiazine has been said to be less toxic in general than sulphanilamide, sulphathiazole and particularly sulphapyridine, with which, in constitution and in range of application, it has much in common (Feinstone, Williams, Wolff, Huntington and Crossley, 1940; Long, 1941; Long, Bliss and Ott, 1941; Meleney, 1943; Plummer and Wheeler, 1944). It has been especially recommended for the treatment of burns (Pickrell, 1941); but the advantages of this have been questioned by Meyer and Gradman (1943). According to Pilcher (1944) it may safely be used locally in brain injuries, whilst sulphathiazole, on account of its causing convulsions, is here definitely contraindicated. It has also been stated that sulphadiazine can be pushed up to higher blood levels than, for example, sulphapyridine without increasing its toxicity (Feinstone *et al.*, 1940; Plummer and Ensworth, 1940). The experiments reported here will have some bearing on this point. Sulphaguanidine, the other drug tested, has, on account of its relatively low absorbability from the alimentary tract, been particularly recommended (Marshall, Bratton, White and Litchfield, 1940) and, in fact, almost exclusively used for the treatment of gastro-intestinal infections, a treatment which in a way can be regarded also as a kind of local drug application.

As these tissue culture experiments were carried out in relation to topical drug administration, rather high drug concentrations (from saturation strength downwards) were tested only.

METHODS.

The cells which were used as test objects in the present study were exclusively macrophages, whose important role in the physiology of wounds is beyond dispute. They were obtained as pure cultures, living in ordinary D-type Carrel flasks, from the

buffy coat of adult hen blood according to Baker's technique (1933). Attached to the bottom of the flask, they form a unicellular layer of isolated individuals, and can be kept alive, healthy and multiplying for many weeks, their feeding fluid being 1 ml. per flask of 20 to 40 per cent hen blood serum diluted with Tyrode solution. For a quantitative study of their rate of multiplication Willmer's (1933) serial photographic method, using 3-in. bromide paper film, has been adapted (Jacoby, 1937). Analysis of the films allows accurate determination of the percentage of cells undergoing mitosis per hour. In the accompanying curves the mean percentage of 2-hour periods has been plotted. Normally, i.e. after serum feeding, there is first a latent period of 20–24 hours, during which hardly any mitoses occur; then a major wave of mitotic activity sets in, lasting for the next 20–24 hours; this may be followed by a minor mitotic wave covering the third day, after which mitotic activity subsides to practically zero. The length of the latent period has been so well established in many standardized serum-experiments that, from reasons of economy, photography was here only started towards the end of that period. The curves b_1 and b_2 in Fig. 1, though representing experimental cultures, show a mitotic response equal to, and thus typical for that of, control cultures.

The procedure adopted was, therefore, as follows: Drug application in 40 per cent. serum at zero hour, i.e. 3 to 4 days after the last feeding. A single test photograph of the chosen field immediately taken for morphological comparison. 18 hours later start of serial photography, which was continued for 40 to 50 hours, then replacement of the drug-containing serum by fresh serum. Only in cases in which gross inhibition and much cell disintegration was evident was the drug removed at an earlier time and fresh serum given (Fig. 4, *a*). Otherwise the drug was left in contact with the cells for three (!) days.

Apart from this growth analysis the films also permit of various important morphological observations; and provided that only extremely healthy cultures showing transparent, freely amoeboid cells are used, and that control cultures in drug-free serum retain this appearance, some of these morphological changes, if occurring in the experimental cultures, can reasonably be taken as signs of impaired health—in other words as toxic effects due to the drug treatment.

Preparation of drug-containing medium.

Weighed quantities of the respective drug were added to measured amounts of 40 per cent serum, kept at 39° C. for 24 hours and frequently shaken. Prior to an experiment the undissolved drug crystals were sedimented by rapid centrifugation and the supernatant drug-saturated serum used for application to the cultures. The dried drug residue was determined, and so information gained about the absolute amount of drug present in each experiment. This procedure proved rather important as the solubility varied considerably with the different sera used, particularly that of sulphadiazine, namely, from 135 mg. per cent to 450 mg. per cent, less that of sulphaguanidine, ranging from about 250 to 400 mg. per cent. For experiments with lower concentrations the drug was dissolved in Tyrode solution at 39° C. and then mixed with serum to give the desired final concentration.

RESULTS.

Sulphadiazine: Saturated solution in 40 per cent serum produced in all experiments a definite depressing effect on the mitotic activity of the macrophages. This is best seen in the mean curve given in Fig. 1, *a*. The hourly percentage mitoses hardly ever rises above 1. The curves of the individual experiments (Fig. 2) show the variations

encountered in the degree of inhibition. This relatively uniform response in inhibition is surprising in view of the widely different absolute amounts of drug present, at least in some of these experiments (Fig. 2). There was, however, a differential effect with regard to the morphology of the cells. In experiments M-S 26, 27, 28 the cells looked perfectly healthy, transparent and amoeboid throughout the course of the test, except perhaps for a certain gradual decrease in size; in experiment M-S 31 the cells were more granulated and showed a definite tendency to round off, which was pronounced on the third day of the drug treatment. Recovery followed quickly the replacement of the drug-containing fluid by fresh serum. In experiments M-S 47 and 53, which had the highest drug content, accumulation of fat droplets and rounding off was somewhat greater still and even disintegration of cells occurred. Fig. 3 (*a*) shows, after

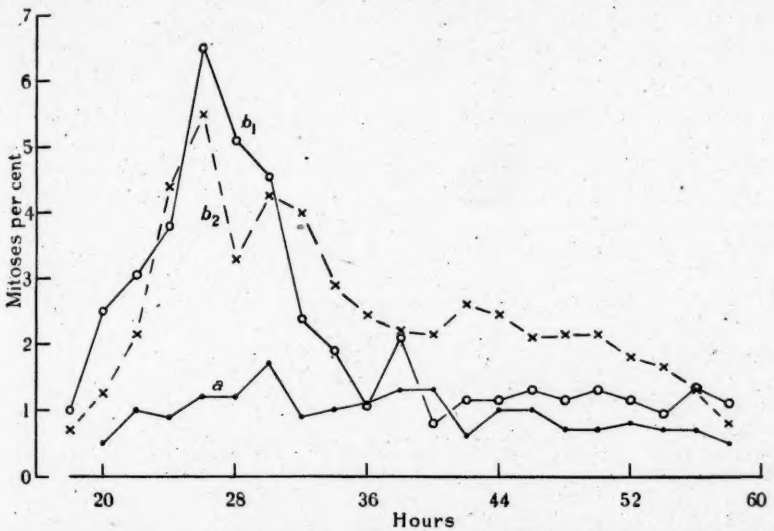


FIG. 1.—Sulphadiazine. a = Saturation strength (mean of 6 experiments); b_1 and b_2 = half saturation strength (M-S 51 and 52).

the slight initial rise in cell population concomitantly with the mitotic activity, the subsequent steep drop due to this disintegration of many cells, most of which were seen to be phagocytosed by neighbouring cells. Recovery of M-S 53 in fresh serum was complete, that of M-S 47 less definite. (See also Figs. 5 and 6.)

It has recently become known that the solubility of sulphadiazine rises sharply in buffer solutions above pH 7 (Gilligan and Plummer, 1943). The pH of the kind of medium used here (hen serum diluted with Tyrode solution) was on the several occasions tested and found to vary between 7·6 and 8·4.* It is therefore possible, and indeed very likely, that the great differences in solubility (a range of 300 per cent.) which were encountered are to some extent explainable by variations in the alkalinity of the medium. But interest should be focused on the fact that in spite of these striking differences in drug content the degree of mitotic inhibition was very similar in all experiments. This seems to indicate that in those experiments with the larger

* A more precise statement on this point cannot be made as the pH of the individual sera actually used for the experiments was not determined. In fact, the experiments were done before the paper of Gilligan and Plummer was available.

amounts of drug, a substantial portion of the drug must have been "inactive." If this contention was true, experiments with half-saturation strength, prepared by dilution from a "highly" saturated serum, should show little or no inhibition of mitotic activity. This was actually found to be the case. In two experiments (M-S 51 and 52), for which a 40 per cent serum with a drug concentration of 450 mg. per cent was used and diluted twofold, there was a vigorous mitotic response equal to controls in drug-free serum (Fig. 1, b_1 and b_2), and not the slightest morphological sign of impaired

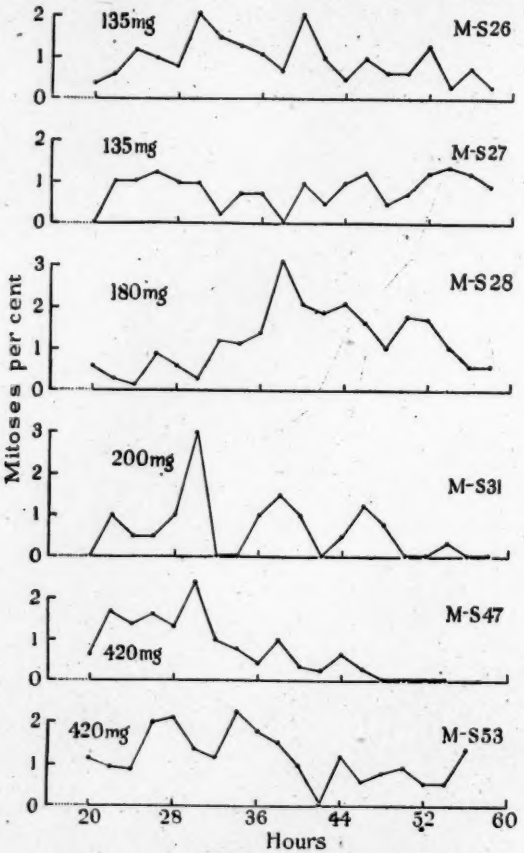


FIG. 2.—Sulphadiazine (saturation strength).

health of the cells; and this in spite of the fact that the final drug concentration was here 225 mg. per cent. The curves should be compared with that given by a saturated 40 per cent serum of similar strength (M-S 31 in Fig. 2).

In a final concentration of 100 mg. per cent mitotic activity following a slightly prolonged latent period was almost normal and the morphology unaltered.

In a concentration of 33 mg. per cent there was no difference from normal cultures as to the rate of multiplication and cell morphology.

Sulphaguanidine: Saturated solution (250 to 400 mg. per cent) in 40 per cent serum. Mitotic activity was almost completely suppressed (Fig. 4, *a*). In addition,

the cells, whilst under drug treatment, showed morphological signs of so seriously impaired health that it was not advisable to prolong the drug application beyond two days. From transparent, slender, amoeboid cells they changed into large flattened

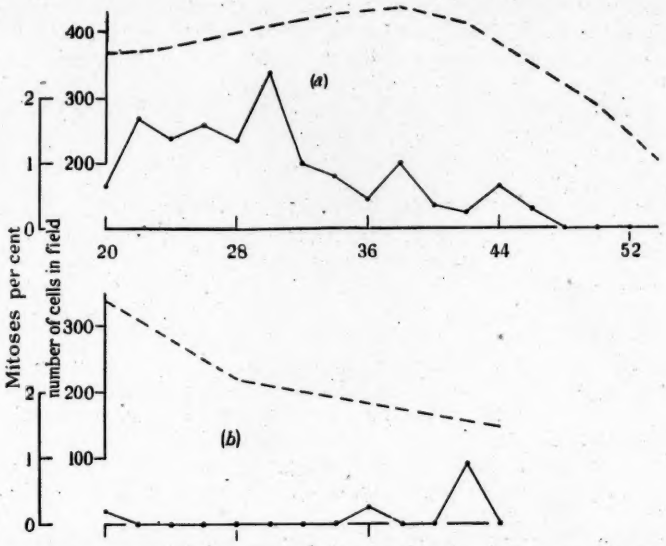


FIG. 3.—(*a*) Sulphadiazine (M-S 47) (saturation strength), 420 mg. per cent; (*b*) sulphaguanidine (M-S 48) (saturation strength), 250 mg. per cent.

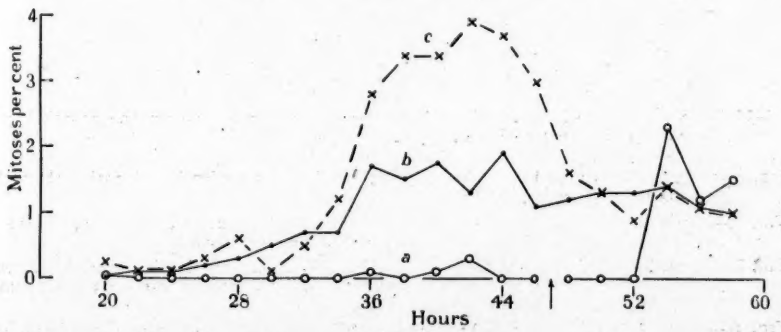


FIG. 4.—Sulphaguanidine. *a* = Saturation strength (250–400 mg. per cent) (mean of 3 experiments); *b* = 100 mg. per cent (mean of 8 experiments); *c* = 33 and 20 mg. per cent (mean of 3 experiments). At ↑ in (*a*) drug-containing serum removed and replaced by fresh serum.

discs, rounded or elongated cells, and all filled with numerous fat droplets. Often many cells would disintegrate and were phagocytosed by fellow cells (see decrease of population in Fig. 3 (*b*) and Fig. 8 as compared with 7). This observation is of special interest, as it shows that one of the most vital functions of these cells, namely, phagocytosis, was by no means abolished. On account of this phagocytosis many of the surviving cells increased considerably in size (Fig. 8). When after two days the drug-containing

medium was replaced by fresh serum mitotic activity was soon resumed (Fig. 4, *a*), and gradually the cultures showed also morphologically complete recovery (Fig. 9).

In a final concentration of 100 mg. per cent there was still a definite inhibition of cell multiplication (Fig. 4, *b*). In most experiments the latent period was considerably lengthened, but was then followed by a wave of mitotic activity which, however, remained below the normal height; in two experiments mitosis started somewhat earlier and also reached a somewhat higher peak. The cells, again, showed a fair amount of fat accumulation in most instances; many would shrink and round off; others often had a peculiar ragged appearance, not seen in normal cultures. Phagocytosis, as described above, was also observed, but not many cells would disintegrate. (Fig. 10 shows macrophages of this series, which, apart from some fat accumulation, appear little affected.)

The variations encountered in the degree of inhibition in this series was reflected in the mode and degree of recovery. In a few experiments there was little or only a very poor recovery in fresh serum following a three-day drug treatment; in others recovery in fresh serum was complete, and in a single instance even a spontaneous morphological recovery occurred, whilst the cells were still exposed to the drug-containing serum. In an experiment with sulphaguanidine in half-saturation strength, equalling a concentration of approximately 110 mg. per cent, the degree of inhibition fell well within the range of that characteristic for the 100 mg. per cent series.

In still lower concentrations (33 mg. per cent and 20 mg. per cent) toxic effects were decidedly less. A prolongation of the latent period to about 30 hours must be considered as significant. But then a strong wave of mitotic activity followed of almost normal height and duration (Fig. 4, *c*). Morphologically, in 33 mg. per cent there was transitorily some rounding off and fat appearance, but spontaneous recovery occurred during the course of the experiments; in 20 mg. per cent the cells remained of practically normal appearance throughout (Fig. 11).

FIGS. 5–11 illustrate the action of sulphadiazine and sulphaguanidine on the morphology of cultures of adult hen blood macrophages. × 130.

FIG. 5.—Expt. M-S 47; 16 hours after application of sulphadiazine in saturation strength in 40 per cent serum. Cells look still almost normal.

FIG. 6.—Same culture (different field); 69 hours after drug application. Some fat accumulation and tendency to round off are noticeable.

FIG. 7.—Expt. M-S 48; 16 hours after application of sulphaguanidine in saturation strength in 40 per cent serum. Cells have a decidedly abnormal appearance; fat accumulation already evident.

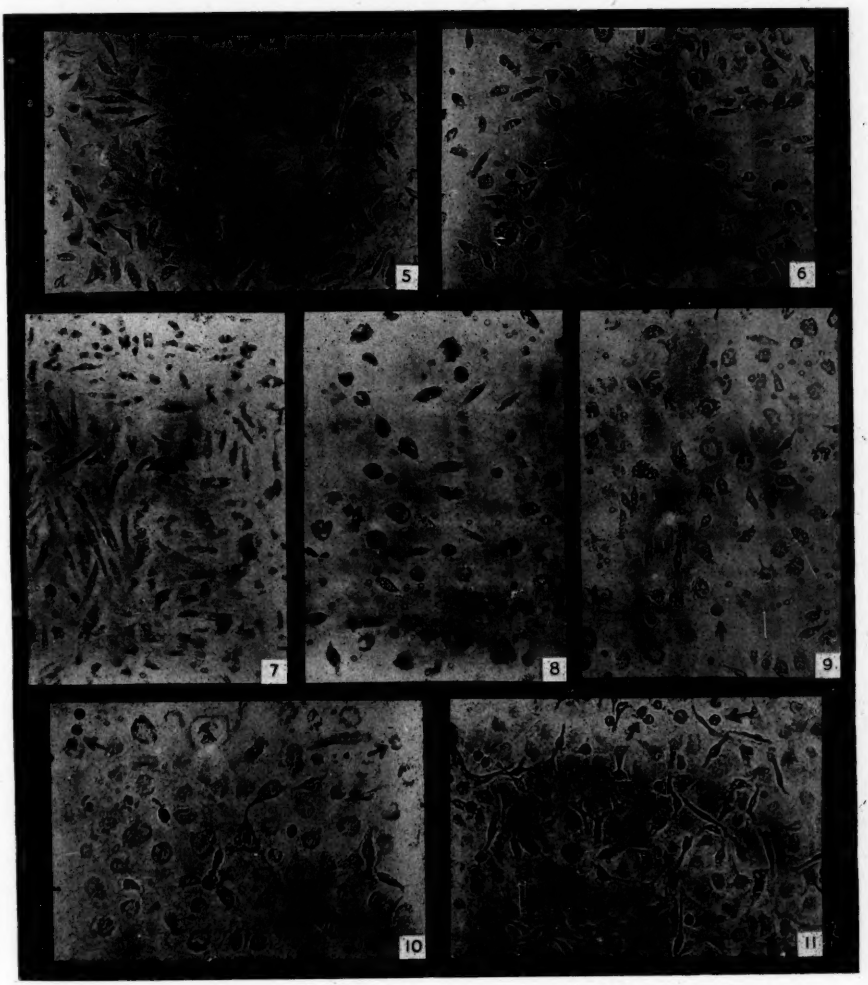
FIG. 8.—Same culture (same field); 44 hours after drug application. Fat accumulation excessive, some rounding off; note also decrease of population and increase in size of many of the surviving cells.

FIG. 9.—Same culture (same field); 44 hours after drug *removal* and now in fresh serum. Recovery well advanced; ↑ points to mitosis.

FIG. 10.—Expt. M-S 36; 45 hours after application of 100 mg. per cent sulphaguanidine in 40 per cent serum. Some fat accumulation, otherwise little change. ↑ points to cell in mitosis. × indicates disintegrating cell being phagocytosed by three neighbouring cells.

FIG. 11.—Expt. M-S 37; 44 hours after application of 20 mg. per cent sulphaguanidine in 40 per cent serum. Normal morphology, numerous mitoses (↑).

Note.—The pictures represent only parts of the fields actually photographed.



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DISCUSSION.

Considering the severe condition under which these cells were held (2 to 3 days' continuous drug treatment in the absence of a circulation), the *relative* harmlessness of the drugs tested stands out as a striking fact. This is in general agreement with the previous work (Jacoby, Medawar, Willmer, 1941). Sulphadiazine in saturation strength is shown to have a definite inhibitory effect on the mitotic activity of macrophages *in vitro*. Pomerat and his co-workers (1944) did not see any inhibition of fibroblasts from heart explants or of leucocytes from splenic tissue under the influence of added crystals of sulphadiazine to the cultures, but they considered migration of cells only and not mitosis. More surprising is that the same authors also found sulphaguanidine under similar condition practically non-injurious, whilst the present work shows this drug, in saturation strength, to cause severe damage to macrophages. A possible explanation of this discrepancy might lie in the different experimental set-up. These workers, apart from the fact that they used a plasma-embryo extract medium which may well contain larger amount of antagonists, added the drug as crystals to the coagulum—a procedure which may not have resulted in anything like saturation strength during the time of observation.

Some comment has already been made on the wide range of solubility of sulphadiazine in different sera which was found to contrast with the relative uniformity of growth inhibition. The available data do not allow an exact analysis of this observation, but any explanation which would tend to put both increased solubility and inactivation on a common denominator has to take two important factors into consideration, viz. the alkalinity of the medium and " binding " of the drug by serum constituents. Though rising alkalinity considerably increases the solubility of sulphadiazine, its bacteriostatic effectiveness—in contrast to that of sulphaguanidine—has been shown to vary inversely with increasing pH above 7 (Brueckner, 1943). This might also hold true for its toxicity towards somatic cells, and would thus, to a large extent, explain the relative lack of increased growth inhibition in the experiments with a very high drug content, assuming that rise in pH was mainly responsible for the latter. Binding of the drug by serum proteins (Davis, 1942, 1943 ; Heinemann, 1943 ; Gilligan, 1943) probably also affects both the inactivation of the drug and its solubility. The values for " bound " drug, as given by these authors, are 55 per cent for sulphadiazine and 25 per cent for sulphaguanidine. Davis produced evidence, in experiments with *E. coli*, that the drug bound in such a way is probably inactive. In addition, it is likely that, with different sera, variations in the concentration of these serum constituents responsible for binding have their share in the differential solubility of the drug. Both these factors may well play a similar role in the environment of wounds, especially if these are treated with fluids like Dakin's or Pickrell's solution, which are fairly alkaline. That binding alone cannot account for the differential toxicity of the two drugs towards macrophages *in vitro* can, for instance, be seen from the experiments represented in Fig. 3. If, here, allowance is made for " binding " on the basis of the above data, the concentrations of the " free " amounts of the respective drugs would work out approximately the same (200 mg. per cent), but there is no doubt that the greater inhibitory and toxic effect must be ascribed to sulphaguanidine.

The more severe morphological damage which became gradually apparent in those sulphadiazine experiments with a very high drug content might find its explanation in the fact that with the gradual breakdown of serum proteins under the digesting action of the cells " bound " drug would be continuously liberated, so to speak, *in statu nascendi*, penetrate the cells and exert additional toxic effects.

Objections have been raised (McIlwain, 1942) against such tissue culture experiments evaluating sulphonamide toxicity on the ground that media like chicken plasma and embryo extract contain unknown amounts of drug-antagonists. This is certainly true, but is has to be remembered that cells *in situ*, exposed to high concentrations of these drugs, as in wound treatment, live actually in a similar antagonist-containing environment. Thus tissue culture studies employing dilute serum, as the present ones do, will give a truer and, in a way, more comparable picture of the direct effects of these drugs on cells *in situ* than would experiments with synthetic antagonist-free media, if such were available for the cultivation of somatic cells. That drug-antagonists of the competitive type may actually intervene in such tissue culture experiments can be shown indirectly by parallel tests employing related sulphone-compounds which, *in vitro*, are said not to be antagonized by p-amino-benzoic acid. Some preliminary experiments with Marfanil* and V 187* clearly revealed a definitely greater toxicity of these new compounds under otherwise closely similar conditions. Macrophages were invariably killed in a concentration of 100 mg. per cent of both these drugs when applied for 20 hours, and still strongly inhibited in a concentration of 20 mg. per cent.

In comparing the toxic effects of sulphonamides on cells *in vitro* with their toxicity in animals, only " acute " toxicity tests can be considered. Feinstone *et al.* (1940), using the more soluble sodium salt of sulphadiazine found, in mice, the lethal blood concentration to be 175–200 mg. per cent, and Marshall *et al.* (1940) give for sulphaguanidine 182 mg. per cent as the lethal blood level. These figures, particularly that of sulphaguanidine, are of an order of magnitude silmilar to the ones presented here. That they are somewhat lower is not surprising if one considers that in so treated animals the acute effects of these drugs will be directed against cells probably more sensitive to them and more vital for the organism than macrophages.

In order to have a quantitative expression for comparing the effects of the various sulphonamide compounds under the condition described, the added hourly percentages of mitoses occurring between the 23rd and 48th hour can usefully be employed. Table I, giving mean figures, summarizes the results obtained with five such drugs. In the first column the figures are related to widely different concentrations, but are important from the point of view of local application. Here sulphanilamide appears as the most toxic, probably on account of its high solubility, which establishes a concentration far exceeding that of the others. In fact, the cells are invariably killed within 20 hours. Osgood (1942) has recorded a similar experience with respect to cultured human bone-marrow cells. Sulphapyridine is practically without effect. At lower, equal, and still inhibitory concentrations the order of toxicity is roughly maintained and, broadly speaking, parallels solubility. The figures give only an approximate picture of the state of affairs, as, for instance, lengthening of the latent period and morphological changes due to drug action are not represented. These do not always coincide with mitotic inhibition (see column " toxic effects on cell morphology " in Table I). Thus, sulphathiazole, in inhibitory doses, produces decidedly more severe morphological changes than does sulphadiazine, the latter, in this respect, being more closely allied to sulphapyridine.

Such excessively high concentrations of the drugs as were obtained, at least, in some of these experiments are unlikely to be reached in wound fluids. Also, the relative size of cell populations *in situ* directly exposed to highest concentrations of the drugs probably exceed those tested in these *in vitro* studies. Both factors will tend to decrease the toxic drug effects on tissue cells *in situ*. A third factor, working in the

* The author is indebted to Dr. W. Short, of Boots, Ltd., for the supply of Marfanil, and to Dr. J. Walker, of the National Institute for Medical Research, Hampstead, for that of V 187 prepared by him.

TABLE I.—*Comparison of the Extent of Cell Multiplication of Macrophages which can take place in Various Concentrations of Five Sulphonamide Compounds. The Figures (averages) Indicate the Sum of Hourly Percentages of Mitoses Occurring Between the 23rd and 48th Hour after Drug Application in Serum.*

	Saturation strength.	400 mg.%.	100 mg.%.	40–28 mg.%.	Toxic effects on cell morphology.
Sulphanilamide	0 (1600 mg.%)	11·0	18·5	65·4	+++
Sulphaguanidine	1·0 (250–400 mg.%)	..	25·8	50·8	++
Sulphadiazine	27·6 (135–450 mg.%)	28·5	43·2	54·1	∓
Sulphathiazole	34·5 (200 mg.%)	..	37·4	67·8	+
Sulphapyridine	50·4 (125 mg.%)	..	61·3 (60 mg.%)	61·3	0

Controls: 50 to 75.

same direction, is the gradual dilution of the concentration due to diffusion and absorption (see Hawking, 1941) and added wound secretion.

From the results of the present work sulphadiazine, being so little harmful to somatic cells, could strongly be recommended for local use, were it not for the unusual degree of "inactivation" which was experienced, at least in some of these tests. If a similar state of affairs should exist in wounds or opened body cavities with a corresponding inhibition of its bacteriostatic activity, this might seriously detract from its value. Sulphaguanidine, on the other hand, seems worth a more extensive trial in local application than has been made so far. It seems as if the fact that it was originally designed as a weapon against intestinal infections has blocked the way to other avenues of application. It is definitely less toxic to somatic cells than sulphanilamide; it is also less soluble and, therefore, less quickly removed from wound surfaces (Hawking, 1941), but it is more soluble than sulphathiazole and sulphadiazine, and will, therefore, spread faster through wounds. Thus its local administration, alone or in combination with either sulphadiazine or sulphathiazole, might prove a favourable procedure in the prophylaxis of wound infections.

SUMMARY.

Sulphadiazine in saturation strength depresses the mitotic activity of macrophages, but affects the morphology of cells little or not at all. Concentrations ranging from 135 mg. per cent to 450 mg. per cent were obtained with different sera, yet the growth inhibition was fairly uniform, indicating "inactivation" of substantial amounts of this drug in some experiments. In 100 mg. per cent and lower concentration the drug is practically without effect.

Sulphaguanidine in saturation strength suppresses almost completely all mitotic activity, and many cells succumb, but recovery of the surviving cells is possible. In 100 mg. per cent cell multiplication is still reduced, and also other morphological signs of impaired health are evident. In still lower concentrations toxic effects are minimal.

Phagocytosis was not suppressed in any concentration of the two drugs.

The toxicity of five sulphonamide compounds, on the basis of their effects on macrophages *in vitro*, is compared, and the relative suitability of some of them for local use is discussed.

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REFERENCES.

BAKER, L. E.—(1933) *J. exp. Med.*, **58**, 575.
BRUECKNER, A. H.—(1943) *Yale J. Biol. Med.*, **15**, 813.
DAVIS, B. D.—(1942) *Science*, **95**, 78.—(1943) *J. clin. Invest.*, **22**, 753.
FEINSTONE, W. H., WILLIAMS, R. D., WOLFF, R. T., HUNTINGTON, E., AND CROSSLEY, M. L. —(1940) *Johns Hopk. Hosp. Bull.*, **67**, 427.
GILLIGAN, D. R.—(1943) *J. Pharmacol.*, **79**, 320.
Idem AND PLUMMER, M. C.—(1943) *Proc. Soc. exp. Biol.*, **55**, 142.
HAWKING, F.—(1941) *Lancet*, i, 786.
HEINEMANN, M.—(1943) *J. clin. Invest.*, **22**, 29.
JACOBY, F.—(1937) *J. Physiol.*, **90**, 23P.
Idem, MEDAWAR, P. B., AND WILLMER, E. N.—(1941) *Brit. med. J.*, ii, 149.
LONG, P. H.—(1941) *J. Amer. med. Ass.*, **116**, 2399.
Idem, BLISS, E. A., AND OTT, E.—(1941) *Johns Hopk. Hosp. Bull.*, **69**, 297.
MARSHALL, E. K., BRATTON, A. C., WHITE, H. J., AND LITCHFIELD, J. T.—(1940)—*Johns Hopk. Hosp. Bull.*, **67**, 163.
MCILWAIN, H.—(1942) *Brit. J. exp. Path.*, **23**, 265.
MELENEY, F. L.—(1943) *Ann. Surg.*, **118**, 171.
MEYER, K. A., AND GRADMAN, R.—(1943) *Surg. Gynec. Obstet.*, **76**, 584.
OSGOOD, E. E.—(1942) *Ibid.*, **75**, 21.
PICKRELL, K. L.—(1941) *Johns Hopk. Hosp. Bull.*, **69**, 217.
PILCHER, C.—(1944) *Ann. Surg.*, **119**, 509.
PLUMMER, N., AND ENSWORTH, H. K.—(1940) *Proc. Soc. exp. Biol.*, **45**, 734.
Idem AND WHEELER, C.—(1944) *Amer. J. med. Sci.*, **207**, 175.
POMERAT, C. M., CAPOUYA, M., AND GREENLEAF, F. I.—(1944) *Texas Rep. Biol. and Med.*, **1**, 97.
WILLEMR, E. N.—(1933) *J. exp. Biol.*, **10**, 323.

THE EFFECT OF TETRATHIONATE ON BACTERIAL GROWTH.*

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TETRATHIONATE broth has been widely used as a routine medium for isolating organisms of the Salmonella group from faeces and other sources (Muller, 1923; Holt, Vaughan and Wright, 1942; Hynes, 1942; Knox, Gell and Pollock, 1942; and others). In an attempt to explain the selective action of the medium, Knox, Gell and Pollock (1943) investigated the effect of its different constituents, and found that though each of these (tetrathionate, thiosulphate, iodide and chalk) had some influence on the overall selectivity, the most important single constituent was the tetrathionate. They found that cultures of *Bact. paratyphosum B*, salmonella and *Proteus* reduced tetrathionate rapidly and quantitatively to thiosulphate, while *Bact. coli*, dysentery bacilli and many other organisms lacked this power. The ability to reduce tetrathionate was also demonstrated in washed suspensions of *Bact. paratyphosum B* (Pollock and Knox, 1943), and was later shown to be of an adaptive nature (Knox and Pollock 1944). It was suggested that tetrathionate acts, for those organisms which can reduce it, as an alternative hydrogen acceptor to oxygen, and thereby provides these, under the conditions of low oxygen tension present, with an additional source of energy not

* A Report to the Medical Research Council.

available to organisms which cannot reduce tetrathionate. It was also suggested that this property in part explains its selective action. Further evidence for this suggestion is given in this paper.

METHODS.

The organisms investigated were several freshly isolated strains of *Bact. paratyphosum B* and one strain of paracolon bacillus ("G.S.M.") which, though unable to reduce tetrathionate, was able to grow well in relatively high concentrations of it. The cultures were maintained on egg slopes from which 18-hour broth cultures were used in experiments. Suitable inocula of these were made into tubes of tryptic digest broth and tetrathionate broth and the rate of growth followed by taking periodic samples, diluting appropriately in phenol (to give a 1 per cent concentration of the disinfectant), and counting the total number of organisms in a Helber counting chamber with dark-ground illumination (Wilson, 1922). To avoid complicating effects due to the selective action of iodides, pure tetrathionate was used in these experiments. It was prepared as previously described (Mellor, 1930; Pollock and Knox, 1943), and was found to contain 2 molecules of water of crystallization with no detectable iodide, thiosulphate, sulphate or sulphite. (For investigation of this I am indebted to Dr. A. Hickling and Dr. M. Elkins, of University College, Leicester.) Reduction of tetrathionate was followed by titration of samples with 0·005 N I_2 (Knox, Gell and Pollock, 1943). It should be noted that the medium used in these experiments, which contained 0·02 M tetrathionate, was quite different from Muller's medium, which contains in addition iodides and a large excess of thiosulphate.

TABLE I.—Bact. paratyphosum B *and Paracolon Bacillus in Broth and Tetrathionate Broth: Total Counts and Iodine Values.*

	Bacterial counts × 10^6 per ml.				Iodine values (ml. 0·005 N I_2 per ml.).				
	Bact. paratyphosum B.		G.S.M.		*Bact. paratyphosum B.*		G.S.M.		Uninoculated control.
Time (hrs.).	Broth.	S_4O_6 broth.	Broth.	S_4O_6 broth.	Broth.	S_4O_6 broth.	Broth.	S_4O_6 broth.	S_4O_6 broth.
0	10·6*	10·6*	10·5*	10·5*	·1	·2	·1	·2	·15
1	32	25	26	25	·1	·2	·1	·2	...
2	59	59	52	51	·1	·4	·1	·2	...
2½	130	130	...	...	·1	·65	·1	·2	·2
3	222	220	94	100	·1	1·4	·1	·3	...
3½	296	372	...	...	·1	2·6	·1	·3	...
4	312	520	286	190	·1	4·0	·1	·35	...
4½	318	630	...	...	·1	5·2	·1	·35	·3
5	318	810	368	220	·1	6·8	·1	·35	...

S_4O_6 = tetrathionate. * Calculated from the amount inoculated from the parent culture.

RESULTS.

A typical experiment is shown in Table I. 18-hour broth cultures of *Bact. paratyphosum B* (Strain No. 1500) and of the paracolon bacillus (Strain G.S.M.) were each inoculated into two tubes, one containing 0·02 M tetrathionate, 0·2 M phosphate buffer and nutrient broth, the other the same concentration of phosphate broth without tetrathionate. The tubes were incubated at 37°; total counts were done at intervals on all the tubes and samples were titrated with 0·005 N I_2. A control tube was also set up containing 0·02 M tetrathionate and the same concentration of phosphate and broth as the other tubes, but no organisms.

The counts show that with *Bact. paratyphosum B* in the presence of tetrathionate the period of rapid increase in cell numbers was prolonged, and that in 5 hours the popu-

lation was about 2½ times the number reached in broth without tetrathionate. With the paracolon bacillus on the other hand growth in tetrathionate broth was slower than in plain broth. The iodine values show that no appreciable reduction of tetra-

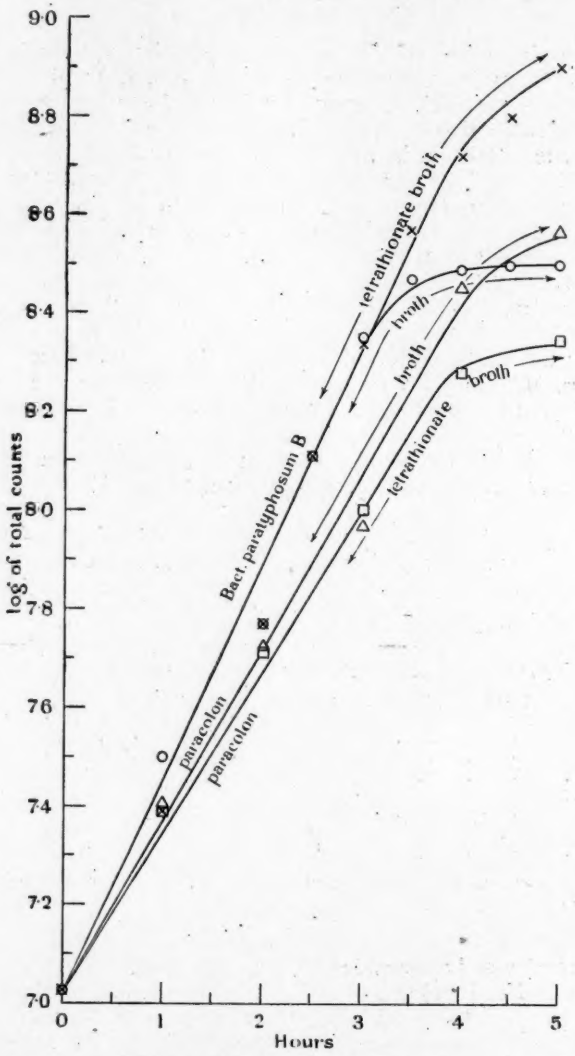

FIG. 1.—Growth of *Bact. paratyphosum B* and paracolon bacillus in broth and tetrathionate broth.

thionate to thiosulphate occurred except in the presence of *Bact. paratyphosum B*, which had begun to reduce it rapidly by 2½ hours and had reduced it nearly all in 5 hours. (Complete reduction of the tetrathionate would give a reading of 8 ml. of 0·005 N I_2 per ml. culture medium.) The early counts were much the same in all four tubes,

and it was not until the bacterial numbers reached about 200 × 10^6 per ml. that any divergence occurred between the tubes with and without tetrathionate. When the logarithms of the bacterial counts are plotted against time (Fig. 1) it can be seen that the effect of tetrathionate on *Bact. paratyphosum B* is to prolong the logarithmic phase of growth. Results of this kind were obtained in several experiments.

A similar effect is obtained by free aeration of broth cultures (Wilson, 1930; Topley and Wilson, 1936; Rahn and Richardson, 1941). Accordingly, three tubes, one con-

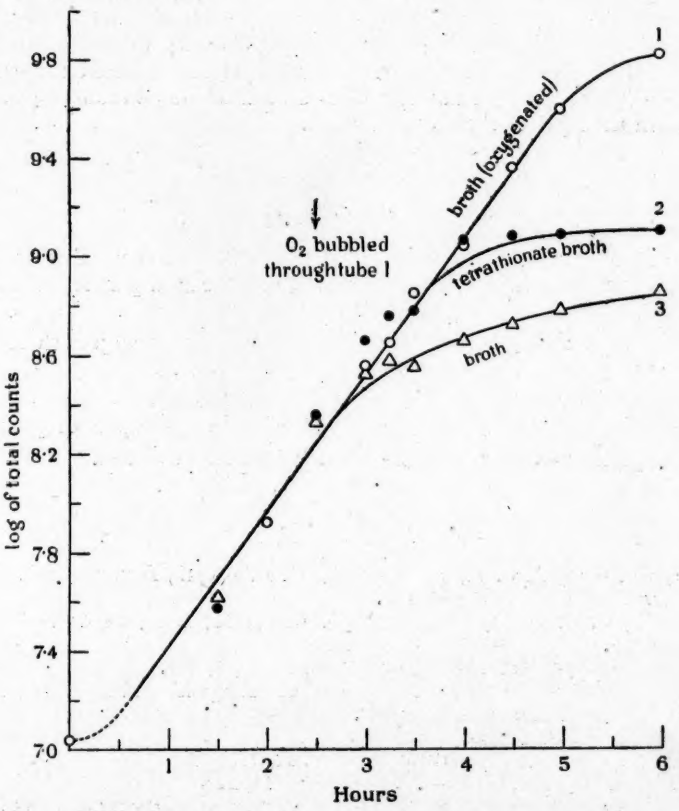

FIG. 2.—Growth of *Bact. paratyphosum B* in broth, oxygenated broth and tetrathionate broth.

taining phosphate tetrathionate broth as before, the other two containing phosphate broth without tetrathionate, received an equal inoculum of *Bact. paratyphosum B* and were incubated in a water-bath at 37°C. After 2½ hours when the total counts had reached 250 × 10^6 per ml. oxygen was bubbled slowly through one of the broth cultures. The growth curves are shown in Fig. 2. The logarithmic phase of growth was prolonged in the tetrathionate broth (long enough to give a 2½–3-fold increase in the count at 4–5 hours), but much more prolonged in the oxygenated broth (long enough to give a 10-fold increase).

DISCUSSION.

These experiments confirm the suggestion previously made that, for organisms capable of reducing it, tetrathionate acts as an alternative hydrogen acceptor to oxygen. The effect of tetrathionate is of the same kind, though not of the same degree, as oxygen, and becomes apparent only when the bacterial population has reached a level at which oxygen is being utilized faster than it can diffuse into the medium. It follows that in mixed cultures of tetrathionate and non-tetrathionate reducers, the reducers have an advantage over the others in the conditions existing in an ordinary unaerated broth culture. The negative aspect of the selective action of tetrathionate (that is, its effect on non-reducers) is as yet unexplained; tetrathionate presumably inhibits non-reducers by blocking some enzyme systems essential for growth. The positive action (that is, the stimulating effect on tetrathionate reducers) can, however, be explained in the light of these experiments.

SUMMARY.

(1) By the addition of tetrathionate (which is reduced to thiosulphate) the growth of *Bact. paratyphosum B* in broth is increased 2 to 3-fold; a paracolon bacillus which cannot reduce tetrathionate is slightly inhibited.

(2) The effect of tetrathionate, like that of oxygen, on the growth of *Bact. paratyphosum B* is to prolong the logarithmic phase of growth.

(3) Experimental support is given to the view that tetrathionate acts, for those organisms which can reduce it, as a specific alternative hydrogen acceptor to oxygen.

(4) This explains in part its selective action in mixed cultures.

REFERENCES.

Holt, H. D., Vaughan, A. C. T., and Wright, H. D.—(1942) *Lancet*, i, 133.
Hynes, M.—(1942) *J. Path. Bact.*, **54**, 193.
Knox, R., Gell, P. G. H., and Pollock, M. R.—(1942) *Ibid.*, **54**, 467.—(1943) *J. Hyg., Camb.*, **43**, 3.
Knox, R., and Pollock, M. R.—(1944) *Biochem. J.*, **38**, 4, 299.
Mellor, J. W.—(1930) 'Comprehensive Treatise on Inorganic and Theoretical Chemistry,' vol. 10. London (Longmans, Green).
Muller, L.—(1923) *C. R. Soc. Biol.*, **89**, 434.
Pollock, M. R., and Knox, R.—(1943) *Biochem. J.*, **37**, 476.
Rahn, O., and Richardson, G. L.—(1941) *J. Bact.*, **41**, 225.
Topley, W. W. C., and Wilson, G. S.—(1936) 'The Principles of Bacteriology and Immunity.' 2nd edition. London (Edward Arnold & Co.).
Wilson, G. S.—(1922) *J. Bact.*, **7**, 405.
Idem.—(1930) *J. Hyg., Camb.*, **30**, 433.

RENAL BLOOD FLOW IN EXPERIMENTAL "CRUSH SYNDROME."

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BYWATERS and Beall (1941) have described a condition, seen chiefly in air-raid casualties, in which compression of a limb for many hours, followed by release, produces renal failure from which the patient may die several days later. In fatal cases the kidneys have shown signs of tubular damage with deposits of material resembling blood pigments in the lumen of the tubules. Eggleton, Richardson, Schild and Winton (1943) have studied the effects of tight bandaging (+ trauma) for 5 hours and longer of the hind limbs of anaesthetized dogs. Following release of the compression there was a fall in carotid arterial blood pressure, and marked reductions of urinary secretion and creatinine clearance. Eggleton (1944) has recorded similar results with limb ischaemia in cats.

Scarff and Keele (1943) have shown that complete ischaemia of the kidney for 1½–2 hours in rabbits is followed by death from renal failure, with tubular damage similar to that seen in the kidneys of fatal cases of the "crush syndrome." It is therefore possible that the kidney damage in this syndrome is due to renal ischaemia, following the release of compression of the injured limb. To test this possibility we have studied renal blood flow in cats following experimental limb compression.

METHOD.

All experiments have been performed on chloralosed cats. Arterial blood pressure was recorded *via* a carotid arterial cannula.

Renal blood flow (R.B.F.) was measured either by the direct method described by Keele and Slome (1943) or by the plethysmograph + venous occlusion method of Brodie and Russell (1905).

In the former method the animal is heparinized and a glass cannula is inserted into the left ovarian vein close to its junction with the left renal vein. The cannula is connected by rubber tubing to one end of a horizontal graduated glass tube, the other end being connected to a volume recorder writing on a moving smoked drum. The glass tube is kept adjusted at a height which just balances the pressure in the renal vein. When the renal vein is occluded for a few seconds, at a point immediately medial to the point of entry of the cannulated ovarian vein, the renal venous blood is shunted along the cannula and recorder system. The trace drawn by the volume recorder enables the rate of flow to be measured in c.c. per min.

The advantages of this method are that the renal blood flow is directly measured without congestion of the kidney, and that only 1 or 2 c.c. of blood are temporarily removed from the circulation. After each reading of R.B.F. this blood is immediately returned to the circulation by temporarily raising the level of the horizontal glass tube.

Two groups of experiments have been performed.

TABLE A.—*Limb Ischaemia Group.*

Exp. 1.
4 hours' ischaemia.

Time after release.	R.B.F. c.c./100 g./min.	B.P. mm. Hg.
Before release (3 readings)	180	150
½ min.	96	130
1½ ,,	96	120
3 ,,	100	120
5½ ,,	96	120
15½ ,,	96	120
25½ ,,	96	120
35 ,,	80	100
50 ,,	54	80
60 ,,	48	70
70 ,,	54	60
130 ,,	57	70
140 ,,	45	65
150 ,,	36	60

Exp. 2.
5 hours' ischaemia.

Time.	R.B.F.	B.P.
Before release	190	170
1 min.	190	150
1¾ ,,	190	140
3 ,,	126	120
3¾ ,,	120	120
4¾ ,,	96	115
6 ,,	96	110
12 ,,	48	100
17 ,,	48	100
20 ,,	52·5	100

Exp. 3.
5 hours' ischaemia.

Time.	R.B.F.	B.P.
Before release	212	170
2 min.	106	125
3 ,,	98	105
6 ,,	98	100
9 ,,	74	90
30 ,,	61	95
32 ,,	50	95
38 ,,	25	70
41 ,,	15	50

Exp. 4.
4 hours' ischaemia.

Time.	R.B.F.	B.P.
Before release	205	150
½ min.	108	130
1 ,,	108	120
5½ ,,	114	120
15½ ,,	108	120
25 ,,	108	120
35 ,,	91	100
50 ,,	62	80
60 ,,	55	70
70 ,,	62	60
130 ,,	67	70
140 ,,	51	65
150 ,,	41	60

Time = Time in minutes after removal of rubber bandages on limbs.
R.B.F. = Renal blood flow in c.c./100 g. of kidney/min.
B.P. = Blood pressure in mm. Hg.

Exp. 5.
4 hours' ischaemia.

Time after release.	R.B.F. c.c./100 g./min.	B.P. mm. Hg.
Before release	140	170
1 min.	100	170
1½ ,,	100	170
5½ ,,	35	125
5¾ ,,	45	135
30 ,,	50	130

Exp. 6.
4 hours' ischaemia.

Time.	R.B.F.	B.P.
Before release	110	117
½ min.	90	100
1½ ,,	90	95
2 ,,	75	90
3 ,,	90	85
4½ ,,	90	85
6½ ,,	80	85
8½ ,,	90	85
10½ ,,	90	85
12¼ ,,	90	85

Animal overheated before release.
Rectal temp. 41·5°C. Hyperpnoea

Exp. 7.
5 hours' ischaemia.

Time.	R.B.F.	B.P.
Before release	150	150
¾ min.	90	140
1 ,,	90	130
1½ ,,	75	125
2 ,,	80	115
3½ ,,	75	95
5¾ ,,	75	90
8¼ ,,	60	80
10½ ,,	75	80
13¼ ,,	75	80
16 ,,	60	80
18 ,,	60	85
20¼ ,,	60	85
23½ ,,	60	85

Exp. 8.
4½ hours' ischaemia.

Time.	R.B.F.	B.P.
Before release	165	180
1 min.	125	150
2 ,,	120	145
2¾ ,,	120	140
4 ,,	110	120
5 ,,	110	110
10 ,,	88	100
11¾ ,,	100	120
12¾ ,,	88	110
14¼ ,,	76	95
16 ,,	72	100
19 ,,	60	85
22 ,,	55	65

Group A. Limb ischaemia (8 experiments).

In this group both hind limbs were tightly compressed by application of rubber bandages from foot to groin. Satisfactory ischaemia was indicated by a fall in temperature of the pads of the feet to room temperature. The bandages were left on for 4–5 hours.

Group B. Haemorrhage (7 experiments).

In this group the animal's limbs were not bandaged. Blood was removed, usually in 10 c.c. volumes, *via* a cannula inserted into one femoral artery.

RESULTS.

Group A. Limb ischaemia.

Blood pressure (B.P.) and renal blood flow (R.B.F.) were measured immediately before and at varying intervals up to 2½ hours after release of the bandages on the limbs. Table A sets out the results.

The statistical analysis of the data was carried out by Dr. E. Lewis-Faning, of the Medical Research Council's statistical staff. Briefly the method employed was as follows :

Where identical readings of blood pressure (B.P.) occurred in the same experiment the average of the corresponding readings for R.B.F. were calculated. Since the initial values were different for each experiment, each series was recalculated using 100 as the initial value for each. By a simple transformation these series can then be presented so as to show the proportional reduction in R.B.F. for a given percentage reduction in B.P. Space does not permit presenting these Tables in full, and it should be sufficient to illustrate the method from experiment 1 of Group A.

Group A, Experiment 1.

Revised series using average R.B.F. values for identical B.P. readings.		Initial values expressed as 100.		Percentage reduction.	
B.P. mm. Hg.	Average R.B.F. c.c./100 g./min.	B.P.	R.B.F.	In B.P.	In R.B.F.
150	180	100	100	0	0
130	96	86·7	53·3	13·3	46·7
120	97	80·0	53·9	20·0	46·1
100	80	66·7	44·4	33·3	55·6
80	54	53·3	30·0	46·7	70·0
70	53	46·7	29·4	53·3	70·6
65	45	43·3	25·0	56·7	75·0
60	45	40·0	25·0	60·0	75·0

During the period of limb ischaemia the B.P. tended to rise and just before release, in 7 of the 8 experiments recorded, was 150–180 mm. Hg. The exception was experiment A.6, in which the cat was overheated before release, and the B.P. was 117 mm. Hg. After release of the bandages both B.P. and R.B.F. began to fall, usually within one minute. The B.P. continued to fall progressively when observed for periods of 12–150 minutes in different experiments. The R.B.F. fell markedly at first (Fig. 1), and subsequently more gradually. The results in a typical experiment are shown graphically in Fig. 2.

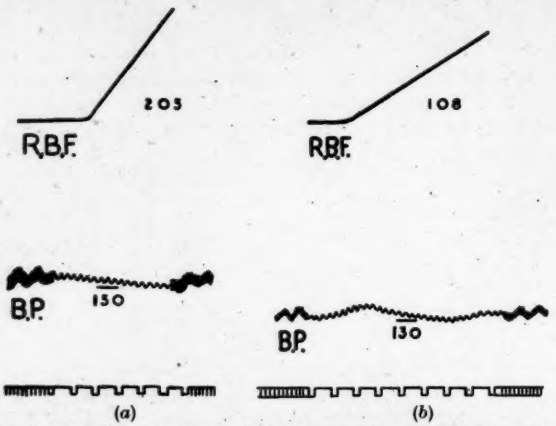

FIG. 1.—Records showing the effect of release of limb ischaemia on Renal Blood Flow (R.B.F.) and Blood Pressure (B.P.) (Experiment 4, Table A). Chloralosed cat. Hind limbs tightly bound with rubber bandages for 4 hours. B.P. by cannula in carotid artery, R.B.F. by venous shunt method.

(*a*) Immediately before removal of bandages:
R.B.F. = 205 c.c./100 g. of kidney/min.
B.P. = 150 mm. Hg.

(*b*) ½ min. after removal of bandages on hind limbs:
R.B.F. = 108 c.c./100 g. of kidney/min.
B.P. = 130 mm. Hg.

Note 47 per cent reduction in R.B.F. associated with a 13 per cent reduction in B.P.

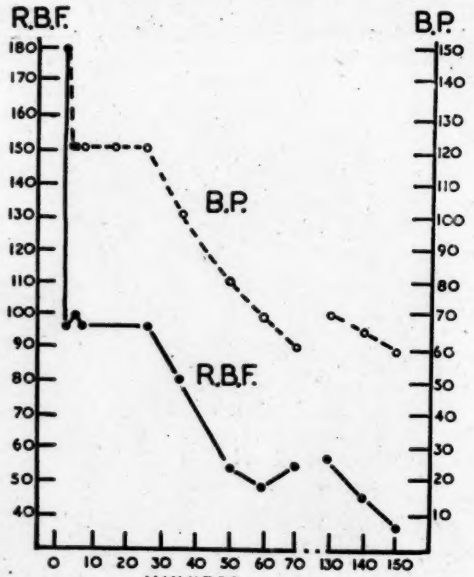

FIG. 2.—Graph showing the effect in a typical experiment (A.1) of release of 4 hours' limb ischaemia on R.B.F. and B.P.

Ordinates: R.B.F. in c.c./100 g. of kidney/min.
B.P. in mm. Hg.

Abscissa: Time after release of ischaemia (min.).

In experiments A.1, 4, 7 and 8 there were substantial reductions in R.B.F. to 46, 47, 50 and 27 per cent of the initial value respectively before the B.P. was reduced 20 per cent. In A.3 there was a 50 per cent reduction in R.B.F. when the B.P. fell 26 per cent below the initial value. In A.5 R.B.F. fell 29 per cent before the B.P. had begun to fall, and when B.P. had fallen 27 per cent the R.B.F. was reduced by 75 per cent. In A.2 there was no fall in R.B.F. in the first 2 min. while the B.P. fell to 18 per cent below its initial value; following this the R.B.F. declined rapidly, so that when B.P. was down to 60 per cent the R.B.F. was reduced to 25 per cent of normal. An example of the initial effect on renal blood flow and B.P. in the first 6 min. following release of compression is shown graphically in Fig. 3 (*a*).

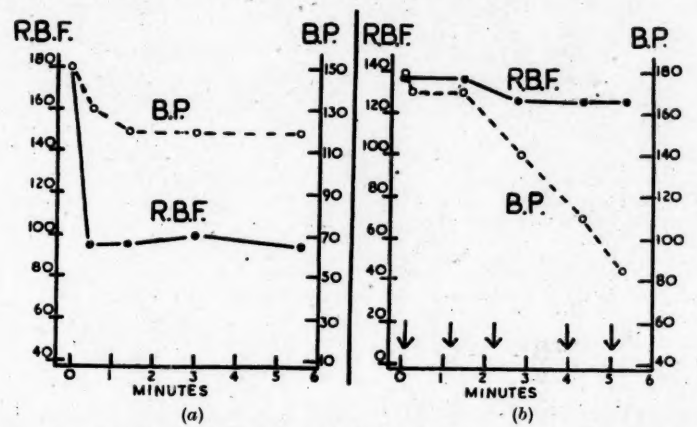

FIG. 3.—Comparison of early effects of release of limb ischaemia and of haemorrhage on R.B.F. and B.P.

(*a*) Graph of R.B.F. and B.P. during first 6 minutes following release of limb ischaemia (Experiment A.1).

(*b*) Graph of R.B.F. and B.P. showing the effect of 5 successive 10 c.c. bleedings (at ↓) (Experiment B.5).

Group B. Haemorrhage.

The experiments of this group were performed to determine how far the reduction in R.B.F. in the ischaemic limb experiments could be attributed merely to the coincidental fall in B.P. In these experiments blood was removed from the femoral artery in amounts sufficient to lower the B.P. to 30–70 mm. Hg. in 10–25 minutes. The results of the experiments are shown in Table B. Fig. 4 shows tracings of the effect on R.B.F. of a reduction of B.P. from 180 to 110 mm. Hg. by haemorrhage (Experiment B.5). A graph of a typical experiment showing the effects of lowering the B.P. by successive haemorrhages on R.B.F. is shown in Fig. 5.

The statistical analysis on Group B was carried out in precisely the same manner as illustrated above for Group A, and from this it was observed that in B.1, 2 and 3 there was no fall in R.B.F. until the B.P. was at least 16 per cent below the initial value. In B.4 and B.5 there was no fall in R.B.F. for a 9 per cent and 6 per cent reduction in B.P., and when the B.P. was reduced to 45 per cent the R.B.F. was still 60 and 88 per cent of normal respectively. In B.6 no reading of R.B.F. was taken till B.P. was reduced to 57 per cent of original level; at this time the R.B.F. was still 80 per cent of the prehaemorrhage value. B.7 was the only case where R.B.F. was reduced for a fall of less than 10 per cent in B.P.

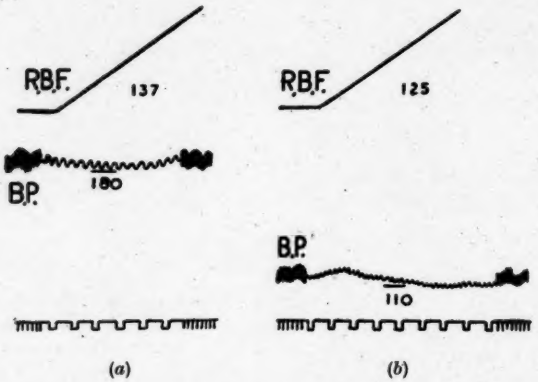

FIG. 4.—Records showing effect on R.B.F. and B.P. of haemorrhage (Experiment B.5). Chloralosed cat: Carotid arterial B.P. R.B.F. by venous shunt. Blood removed from femoral artery.
(*a*) Tracing taken immediately before haemorrhage:
B.P. 180 mm. Hg. R.B.F. 137 c.c./100 g. of kidney/min.
(*b*) Tracing taken 4½ min. later, after 4 separate bleedings of 10 c.c. each:
B.P. 110 mm. Hg. R.B.F. 125 c.c./100 g. of kidney/min.
Note that R.B.F. fell only 9 per cent although B.P. was reduced 53 per cent.

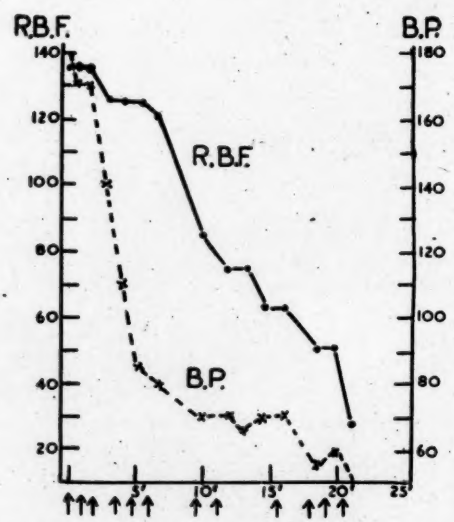

FIG. 5.—Graph showing the effect on R.B.F. and B.P. of successive haemorrhages (Experiment B.5).
Ordinates: R.B.F. in c.c./100 g. of kidney/min.
B.P. in mm. Hg.
Abscissa: Time in minutes.
At ↑ 10 c.c. of blood removed from femoral artery.

TABLE B.—*Haemorrhage Group.*

Exp. 1.

Time.	R.B.F.	B.P.
0	170	155
(10 c.c. haem.)		
2 min.	170	110
3½ min.	170	140
(10 c.c. haem.)		
3¾ min.	170	140
(8·5 c.c. haem.)		
7½ min.	110	75
8¼ „	115	80
9¼ „	135	85
(10 c.c. haem.)		
10 min.	90	60
10¼ „	80	50
11½ „	90	55
(10 c.c. haem.)		..
12 min.	55	45
13½ „	65	50
15 „	50	60
(8 c.c. haem.)		
16 min.	25	40
16¼ „	10	30

Exp. 2.

Time.	R.B.F.	B.P.
0	142	180
(10 c.c. haem.)		
¾ min.	142	140
(10 c.c. haem.)		..
1½ min.	142	100
(10 c.c. haem.)		..
2½ min.	105	70
3½ „	115	70
4½ „	150	80
6½ min.	142	90
(10 c.c. haem.)		..
8 min.	105	80
10 „	105	70

Exp. 3.

Time.	R.B.F.	B.P.
..	165	120
..	165	100
..	137·5	80
..	100	60
..	55	40

Exp. 4.

Time.	R.B.F.	B.P.
..	165	110
..	165	100
(10 c.c. haem.)		
..	100	50
..	120	80
..	127	85
(10 c.c. haem.		
..	91	50
..	120	80
(10 c.c. haem.)		
..	88	55

Exp. 5.

Time.	R.B.F.	B.P.
0	137	180
(10 c.c. haem.)		
¼ min.	137	170
(10 c.c. haem.)		
1½ min.	137	170
(10 c.c. haem.)		
2¼ min.	125	140
(10 c.c. haem.)		
4¼ min.	125	110
(10 c.c. haem.		
5½ min.	125	85
(10 c.c. haem.)		
6¾ min.	120	80
(10 c.c. haem.		
10 min.	85	70
(10 c.c. haem.)		
12 min.	74	70
13 „	74	65
14½ „	63	70
(10 c.c. haem.)		
16 min.	63	70
(10 c.c. haem.)		
18½ min.	51	55
(10 c.c. haem.)		
19¾ min.	51	
(10 c.c. haem.)		
21 min.	28	45
25 „	17	30

Exp. 6.

Time.	R.B.F.	B.P.
0	130	140
(10 c.c. haem.)		
¼ min.	110	80
½ „	118	90
3 „	118	90
(5 c.c. haem.)		
4 min.	97	80
(5 c.c. haem.)		
4¾ min.	67	65
5¾ „	67	70
6¼ „	85	90
(10 c.c. haem.)		
8¼ min.	47	60
9 „	38	60

Exp. 7.

Time.	R.B.F.	B.P.
0	152	130
(10 c.c. haem.)		
1 min.	140	120
(10 c.c. haem.)		
2½ min.	120	100
3½ „	132	110
(10 c.c. haem.)		
6 min.	104	80
7 „	115	90
8 „	120	95
(10 c.c. haem.)		
9 min.	75	50
10 „	88	70
(10 c.c. haem.)		
12 min.	55	40
13 „	45	40

Differences in B.P. and R.B.F. relationship in Group A (limb ischaemia) and Group B (haemorrhage).

The differences between Groups A and B are summarized thus :

1. In 5 of 7 instances in *Group A* the R.B.F. was substantially reduced before the B.P. was reduced 20 per cent. In one further instance this was also probably true. In 3 of 7 instances in *Group B* R.B.F. was unchanged until the B.P. was at least 16 per cent below the initial value. Three other instances probably showed the same relation. One instance in each group is atypical. This contrast between the two groups of experiments is well seen by comparing Fig. 3 (*a*) with Fig. 3 (*b*) and Fig. 2 with Fig. 5.

2. In *Group A* (except in the first two items of Experiment 2) the values of B.P. expressed as percentages of the initial values are higher than the corresponding readings

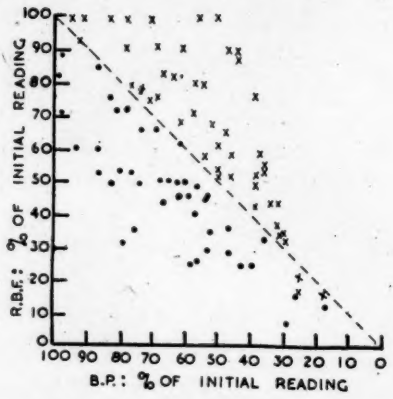

FIG. 6.—Diagram showing relation between R.B.F. and B.P. in limb ischaemia and haemorrhage experiments.
Ordinate : R.B.F. as per cent of initial reading.
Abscissa : B.P. as per cent of initial reading.
● = Readings from limb ischaemia experiments.
× = Readings from haemorrhage experiments.
(It follows from this diagram that in nearly all cases after limb ischaemia the per cent reduction in R.B.F. is greater than the per cent reduction in B.P., whereas after haemorrhage the converse is generally true.)

of R.B.F. expressed as percentages of *their* initial readings ; but in *Group B* the reverse is true (see Fig. 6). Put another way, in *Group A* the per cent reduction in R.B.F. was greater than the per cent reduction in B.P., but in *Group B* the per cent reduction in R.B.F. was less than the per cent reduction in B.P.

Dr. Lewis-Faning concludes that although the data are not adequate for detailed analysis, " from a mere consideration of the type of curve required to fit the two sets of data,* it is highly suggestive that the relation between blood pressure and renal blood flow is different after limb ischaemia to what it is when B.P. is reduced by bleeding. Owing to the fact that two instances are atypical, one cannot be dogmatic about this, but since after limb ischaemia the fall in R.B.F. is more than proportional to the fall in B.P., whilst the reverse is true in the haemorrhage series, and since this holds even in the two experiments which seem to be of the opposite type, we can, I think, consider the difference proved."

* A cubic curve fits the ischaemia group, but a logistic curve is required for the haemorrhage group.

DISCUSSION.

In cats, following the return of the circulation to limbs previously rendered ischaemic for 4–5 hours by tight bandaging, there is a fall in B.P. and a reduction in R.B.F. If the B.P. is reduced to the same extent by other means, e.g. by haemorrhage, the reduction in R.B.F. is proportionately less than after release of limb ischaemia.

Eggleton *et al.* (1943) have shown that release of prolonged limb ischaemia in dogs is followed by a fall in B.P. and marked reductions of creatinine and urea clearances. Eggleton (1944) has also found the same type of response in cats. There is a close correspondence between her figures for creatinine clearance and the results we have obtained with renal blood flow measurements in similar conditions. For example, in one experiment quoted by Eggleton, when the limbs were released after 4 hours' ischaemia, the B.P. fell from 140 to 90–100 mm. Hg. and creatinine clearance was reduced from 12·5 to 4·0 c.c. per min. (i.e. to 32 per cent of the initial value). In one of our limb ischaemia experiments (No. 4), when the B.P. fell from 150 to 80 mm. Hg. the R.B.F. fell from 205 to 62 c.c./100 g./min. (i.e. to 30·2 per cent of the initial value). In Eggleton's cat experiments the average decrease in creatinine clearance was 49 per cent after 4–5 hours of limb ischaemia. These figures agree very closely with our figures for reduction of renal blood flow. We therefore suggest that the reduction in creatinine clearance in Eggleton's experiments may be due to a reduction in renal blood flow.

Our experiments also show that after release of limb ischaemia the reduction in R.B.F. is not due solely to the associated fall in B.P., since a proportionate reduction in B.P. induced by haemorrhage does not reduce R.B.F. as much as release of ischaemic limbs. The mechanisms of the additional factor are at present being studied.

SUMMARY AND CONCLUSIONS.

Following release of 4–5 hours of complete limb ischaemia in anaesthetized cats there is a fall in blood pressure and a marked reduction of renal blood flow. When the blood pressure is reduced to the same extent by haemorrhage there is a smaller reduction in renal blood flow than with limb ischaemia. The reduction in renal blood flow following release of ischaemic limbs may be, in part at least, responsible for the renal tubular damage of the " crush syndrome " type.

We should like to thank Dr. E. Lewis-Faning of the Medical Research Council's statistical staff for analysing our data, and Prof. Samson Wright for his constant encouragement. We are grateful to the Medical Research Council for a grant for technical assistance.

REFERENCES.

Brodie, T. G., and Russell, A. E.—(1905) *J. Physiol.*, **32**, 47.
Bywaters, E. G. L., and Beall, D.—(1941) *Brit. med. J.*, i, 427.
Eggleton, M. G.—(1944) *Lancet*, ii, 208.
Idem, Richardson, K. C., Schild, H. O., and Winton, F. R.—(1943) *Quart. J. exp. Physiol.*, **32**, 89.
Keele, C. A., and Slome, D.—(1943) *J. Physiol.*, **102**, 11 P.
Scarff, R. W., and Keele, C. A.—(1943) *Brit. J. exp. Path.*, **24**, 147.

THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTIBACTERIAL ACTIVITY. PART II: A GENERAL SURVEY OF THE ACRIDINE SERIES.

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In a previous comunication (Rubbo, Albert and Maxwell, 1942) it was established that the antibacterial activities of proflavine, the five mono-aminoacridines and acridine were roughly parallel to their strengths as bases, and the opinion was expressed that a high degree of basicity would probably be found to be an essential attribute for marked bacteriostasis in the acridine series. At the same time it was pointed out that the active compounds owed their high basicity to a special electronic arrangement known as "Extra Ionic Resonance," which can occur in acridine only when an amino group is present in the 2- or 5-position of the acridine ring. Consequently, it was left an open question whether active bacteriostasis was due (*a*) to high basicity *per se* or (*b*) to the real cause of this increased basicity, viz. extra ionic resonance, or (*c*) to a combination of both effects.

The aim of the present work has been to extend the above investigation to a representative cross-section of acridine derivatives with a view to discovering the physical and chemical basis for the antibacterial action of these substances, and to formulate physico-chemical standards with which to predict activity in this series.

The results of this investigation show quite clearly that *the most essential property for marked antibacterial action is a basicity sufficiently high to effect at least* 60 *per cent ionization at pH* 7·3 (37° C.). On the other hand, the activity of an aminoacridine, no less than that of any other drug, may be modified, advantageously or disadvantageously, by alteration of structure and of certain physico-chemical characteristics of the molecule. The conclusions described herein have been reached by the re-examination of 30 typical examples of the 130 acridines whose antibacterial activities have been determined previously in the literature and by the synthesis of 77 completely new compounds.

Apart from the fundamental objectives of this work, we have attempted to direct attention to the clinical possibilities of some new aminoacridines.

MATERIAL AND METHODS.

Chemical.

The methods of preparation and melting-points of the 107 acridines synthesized for this work are given in Table I.

TABLE I.—*The Preparation of the Compounds Used in This Survey.*

Substance.	M. pt. °C.	Preparation.
Acridine	110	British Drug Houses, recrystallized from dilute alcohol.
Monoaminoacridines.		
1-Aminoacridine	106	Albert and Ritchie (1941), *J.S.C.I.*, **60**, 120.
2-Aminoacridine	218	Ditto
3-Aminoacridine	214	,,
4-Aminoacridine	180	,,
5-Aminoacridine (acramine yellow; monacrin)	234	*Idem* (1942), *Organic Synth.*, **22**, 5.
Diaminoacridines.		
1 : 5-Diaminoacridine	—	Attempts to synthesize this have so far been unsuccessful.
2 : 5-Diaminoacridine	(dec.)	Albert and Gledhill (1942), *J.S.C.I.*, **61**, 159.
2 : 6-Diaminoacridine	214–6	Albert and Linnell (1936), *J.C.S.*, 88.
2 : 7-Diaminoacridine (acramine red)	350 (dec.)	Australian Pat. 115,480; Gt. Britain 15,589/41 to University of Sydney.
2 : 8-Diaminoacridine (proflavine)	277	Albert (1941), *J.C.S.*, 121 and 484.
2 : 9-Diaminoacridine	241	Albert and Linnell (1936), *J.C.S.*, 1614.
1 : 9-Diaminoacridine	177	Lahey, unpublished.
3 : 7-Diaminoacridine	303	Bogert, Hirschfelder and Lauffer (1930), *Coll. Czech. Chem. Comm.*, **2**, 383.
3 : 5-Diaminoacridine	229	Albert and Ritchie (1943), *J.C.S.*, 458.
4 : 5-Diaminoacridine	—	Attempts to synthesize this have so far been unsuccessful.
Substituted 5-Aminoacridines.		
5-Amino-1-methylacridine	192–3	Albert and Gledhill, in the press (action of ammonium carbonate on 5-chloro-1-methylacridine).
5-Amino-2-methylacridine	217	Albert and Gledhill, unpublished (as last).
5-Amino-3-methylacridine	241	Ditto.
5-Amino-4-methylacridine	243	,,
5-Amino-1 : 3-dimethylacridine	167	,,
5-Amino-1 : 9-dimethylacridine	136	,,
5-Amino-1-methoxyacridine	241–2	Albert and Gledhill, unpublished (action of ammonium carbonate on 5-chloro-1-methoxy-acridine).
5-Amino-2-methoxyacridine	194–5	Ditto.
5-Amino-3-methoxyacridine	225	Drozdov and Cherntzov (1935), *J. gen. Chem. Moscow*, **5**, 1736.
5-Amino-4-methoxyacridine	273	Albert and Gledhill, unpublished (as 1- and 2-isomerides).
5-Amino-1-hydroxyacridine	244 (dec.)	Albert and Gledhill, unpublished (action of hydrobromic acid on corresponding methoxy compound).
5-Amino-2-hydroxyacridine	over 360	Ditto.
5-Amino-3-hydroxyacridine	178	,,
5-Amino-4-hydroxyacridine	(dec.)	,,
5-Amino-1-chloroacridine	223–4	Albert and Gledhill, unpublished (action of ammonium carbonate on 1 : 5-dichloroacridine).
5-Amino-2-chloroacridine	263	Albert, Dyer and Linnell (1937), *Quart. J. Pharm.*, **10**, 649.

TABLE I (*continued*).

Substance.	M. pt. °C.	Preparation.
5-Amino-3-chloroacridine	274–5	Albert and Gledhill, unpublished (as 1-chloro-isomeride).
5-Amino-4-chloroacridine	261	Ditto.
5-Amino-1-nitroacridine	193	Albert and Gledhill, unpublished (action of ammonium carbonate on 5-chloro-1-nitro-acridine).
5-Amino-2-nitroacridine	290–5 (dec.)	Albert and Gledhill (1942), *J.S.C.I.*, **61**, 159.
5-Amino-3-nitroacridine	300 (dec.)	Albert and Ritchie (1943), *J.C.S.*, 458.
5-Amino-4-nitroacridine	223 (dec.)	Albert and Gledhill, unpublished (as 1-nitro-isomeride).
5-Amino-1-phenylacridine	210–1	Albert and Gledhill, unpublished (action of ammonium carbonate on 5-chloro-1-phenyl-acridine).
5-Amino-3-phenylacridine	215–6	Ditto.
5-Amino-3-cyanoacridine	300–1	Albert and Gledhill, unpublished (action of ammonium carbonate on 5-chloro-3-cyano-acridine).
5-Aminoacridine-1-carboxylic acid	315 (dec.)	Albert and Gledhill, unpublished (as 3-carboxylic acid, below).
5-Aminoacridine-3-carboxamide	312	Albert and Gledhill, unpublished (action of ammonium carbonate on 5-chloroacridine-3-carboxamide).
5-Aminoacridine-3-carboxylic acid	(dec.)	Albert and Gledhill, unpublished (action of ammonium carbonate on 5-chloroacridine-3-carboxylic acid).
5-Amino-3-carbomethoxyacridine (methyl ester of above)	(dec.)	Albert and Gledhill, unpublished (action of ammonium carbonate on 5-chloro-3-carbo-methoxyacridine).
5-Amino-6 : 7 : 8 : 9-tetrahydroacridine	179–80	Albert and Gledhill, unpublished (action of ammonium carbonate on 5 chloro-6 : 7 : 8 : 9-tetrahydroacridine).
5-Amino-2-nitro-7-ethoxyacridine	310	Albert and Gledhill (1942), *J.S.C.I.*, **61**, 159.
5-Amino-2-chloro-7-methoxyacridine	271	Albert, Goldacre and Heymann (1943), *J.C.S.*, 651.
5-Methylaminoacridine	173–4	Albert and Ritchie (1943), *J.C.S.*, 454.
N : N′-*bis*(5-acridyl)ethylenediamine	229	Albert and Gledhill, unpublished (action of 5-chloroacridine on ethylenediamine).
5-Butylaminoacridine	100–2	Albert, Goldacre and Heymann (1943), *J.C.S.*, 651.
5-*cyclo*-Hexylaminoacridine	170–1	Ditto.
5-Heptylaminoacridine (+ HCl)	138	,,
5-Dodecylaminoacridine	67	,,
5-Hexadecylaminoacridine	66	,,
5-(β-Hydroxyethyl) aminoacridine	203	Albert and Gledhill, unpublished (action of ethanolamine on 5-chloroacridine).
2-Chloro-5-(4′-diethylamino-1′-methyl) butylamino-7-methoxyacridine (Mepacrine or atebrin) (+ 2HCl)	243–4	German Patent 553,072 as modified in the University of Sydney).
Ditto (free base)	83–5	
Substituted 2- and 3- Aminoacridines.		
3-Amino-5-methylacridine	210–1	Sharp, Sutherland and Wilson (1943), *J.C.S.*, 344 (modified).
3-Amino-8-chloroacridine	267–9	Bradbury and Linnell (1942), *J.C.S.*, 377.
2-Amino-7-chloroacridine	240 (dec.)	Bradbury and Linnell (1942), *Quart. J. Pharm.*, **15**, 31.
2-Amino-8-chloroacridine	220	Bradbury and Linnell (1942), *J.C.S.*, 377.
2-Aminoacridine-7-carboxylic acid	(dec.)	Albert and Goldacre (1943), *J.C.S.*, 454.

TABLE I (*continued*).

Substance.	M. pt. °C.	Preparation.
2-Amino-7-carbomethoxyacridine (methyl ester of above)	280	Albert, unpublished (esterification of last named).
2-Aminoacridine-7-sulphonic acid	(dec.)	Aarons and Albert (1942), *J.C.S.*, 183.
2-Aminoacridine-7-sulphonamide	253 (dec.)	Ditto.
2-Amino-5-*p*-aminophenylacridine (Phosphine)	270–3	Dunstan and Hewitt (1906), *J.C.S.*, **89**, 483.
Substituted Diaminoacridines.		
2-Dimethylamino-7-aminoacridine	242	Albert, unpublished (as 2 : 7-diaminoacridine).
2 : 5-Diamino-7-ethoxyacridine (Rivanol)	226	Albert and Gledhill (1942), *J.S.C.I.*, **61**, 159.
2 : 8-Diamino-1 : 9-dimethylacridine	170	Albert and Magrath, *J.S.C.I.*, in the press.
2 : 8-Diamino-3 : 7-dimethylacridine	325	Ullmann (1901), *Berichte*, **34**, 4308.
2 : 8-Diamino-4 : 6-dimethylacridine	294	Albert, unpublished (as proflavine).
2 : 8-Diamino-3 : 7-dichloroacridine	(dec.)	Albert (1939), *J.C.S.*, 920.
2 : 8-Diamino-3 : 7-dimethyl-5-phenylacridine (benzoflavine)	307 (dec.)	Meyer and Gross (1899), *Berichte*, **32**, 2360.
2 : 8-*bis*-Dimethylaminoacridine (acridine orange)	181–2	Biehringer (1896), *J. prakt. Chem.*, **54**, 243.
2 : 8 : 2′ : 8′-Tetra-amino-5 : 10-dihydrodiacridyl-5 : 5′-ether	260 (dec.)	Albert (1941), *J.C.S.*, 121.
Quaternary Acridines.		
2-amino-10-methylacridinium bromide	236	Albert and Ritchie (1943), *J.C.S.*, 458.
3-Amino-10-methylacridinium bromide	(dec.)	Ditto.
4-Amino-10-methylacridinium bromide	267	,,
5-Amino-10-methylacridinium bromide	305 (dec.)	,,
5-Imino-10-methylacridane	134–6	,,
3-Amino-5 : 10-dimethylacridinium bromide	259–60	Albert, unpublished (as corresponding chloride : Sharp, Sutherland and Wilson (1943), *J.C.S.*, 344).
2 : 8-Diamino-10-methylacridinium chloride (purified euflavine ; purified neutral acriflavine)	(dec.)	Hall and Powell (1934), *Quart. J. Pharm.*, **7**, 522.
10-Methylacridinium chloride	177 (dec.)	Kaufmann and Albertini (1909), *Berichte*, **42**, 1999.
10-Methylacridinium bromide	(dec.)	Ditto.
Substituted Acridines.		
1-Methylacridine	90	Lehmstedt (1936), *Berichte*, **69**, 2399.
3-Methylacridine	133	Kahn (1894), *Annalen*, **279**, 275.
5-Methylacridine	117	Jensen and Rethwisch (1928), *J. Amer. chem. Soc.*, **50**, 1144.
1 : 3 : 4 : 6 : 7 : 9-Hexamethylacridine	217	Senier and Compton (1907), *J.C.S.*, **91**, 1934.
Ω-Amino-3-methylacridine	(dec.)	Albert, unpublished ; ring-closure of Ω-Amino-4′-methyldiphenylamine-2-carboxylic acid (from the corresponding nitrile and Raney-nickel) with sulphuric acid, and reduction with sodium amalgam.
Ω-Amino-5-ethylacridine	145	Jensen and Howland (1926), *J. Amer. chem, Soc.*, **48**, 1988.
p-Amino-5-styrylacridine	242	Sharp, Sutherland and Wilson (1943), *J.C.S.*, 5.
1-Methoxyacridine	134	Jensen and Rethwisch (1928), *J. Amer. chem. Soc.*, **50**, 1144.
3-Methoxyacridine	105	Lehmstedt (1936), *Berichte*, **69**, 2399.
2 : 8-Dimethoxyacridine	138	German Patent 392,066, to Cassella.

TABLE I (*continued*).

Substance.	M. pt. °C.	Preparation.
Acridine-1-carboxylic acid	190	Albert and Gledhill, unpublished (reduction of corresponding acridone).
Acridine-3-carboxylic acid	(dec.)	Ditto.
Acridine-5-carboxylic acid	(dec.)	Homberger and Jensen (1926), *J. Amer. chem. Soc.*, **48**, 800.
5-Carbomethoxyacridine (methyl ester of above)	128	Jensen and Rethwisch (1928), *J. Amer. chem. Soc.*, **50**, 1145.
Acridine-3-sulphonic acid	(dec.)	Matsumura (1935), *J. Amer. chem. Soc.*, **57**, 1533.
1-Acetamidoacridine	117	Clemo, Perkin and Robinson (1924), *J.C.S.*, 1781.
2-Acetamidoacridine	229	Albert and Linnell (1936), *J.C.S.*, 1614.
3-Acetamidoacridine	230	Lehmstedt (1938), *Berichte*, **71**, 814.
4-Acetamidoacridine	230	Albert and Goldacre (1943), *J.C.S.*, 454.
5-Acetamidoacridine	266	Ditto.
1-Hydroxyacridine	116	Jensen and Rethwisch (1928), *J. Amer. chem. Soc.*, **50**, 1144.
2-Hydroxyacridine	285	Albert and Ritchie (1943), *J.C.S.*, 458.
3-Hydroxyacridine	279 (dec.)	Ditto.
4-Hydroxyacridine	250 (dec.)	,,
5-Hydroxyacridine (acridone)	350	Allen and McKee (1939), *Organic Synth.*, **19**, 6.
3-Aminoacridane	184–5	Albert and Ritchie (1943), *J.C.S.*, 458.

J.C.S. = *J. chem. Soc.* *J.S.C.I.* = *J. Soc. chem. Ind., Lond.*

These substances, for purposes of biological testing, were dissolved in water with the aid of one to one-and-a-half equivalents of hydrochloric or acetic acid. The biological results in the Tables are recorded in terms of the actual base; this procedure has eliminated errors due to varying amounts of water-of-crystallization and to acid radicals of varying molecular weight.

The percentage ionization has been calculated from the Mass Law Equation—

$$\text{Percentage ionized} = \frac{100}{1 + \text{antilog}\,(\text{pH}-\text{pK}_a)}$$

Here, pK_a stands for the negative logarithm of the "acidic dissociation-constant," and is numerically equal to the pH value at which the base is 50 per cent ionized. The pK_a values were obtained by potentiometric titration with the glass electrode (Albert and Goldacre, 1943, and unpublished). Appropriate corrections (e.g. for hydrolysis) were applied where necessary, and wherever possible the titrations were carried out in water. When the solubility was not great enough in this solvent the titrations were effected in 50 per cent ethanol or in 67 per cent methanol. In such cases an addition of 0·5 unit has been made to the pK_a values as a solvent correction. This addition was derived from the work of Mizutani (1925) and from our own experience, and it is believed that this approximation introduces less error than the attempted titration of the less soluble bases in water, as reported by Albert, Rubbo and Goldacre (1941).

Bacteriological.

The same general technique in performing bacteriostatic tests has been followed in this work as in our previous investigation (Rubbo, Albert and Maxwell, 1942).

Medium.

This was prepared by Wright's method, using fat-free minced bullock's heart and peptone of Parke-Davis & Co. The broth was autoclaved for 20 minutes at 15 lb. pressure at pH 8·4–8·8, then filtered, the pH adjusted to 7·4 and re-autoclaved. Ten per cent sterile unheated ox serum was added aseptically and the pH re-determined. The final pH lay between 7·2–7·4 as determined by the glass electrode. This medium was distributed for a bacteriostatic test into sterile 6 oz. bottles in 25 ml. quantities (measured by pipette) and incubated before use for 24 hours at 37° C. in order to test for sterility.

Organisms.

The organisms used in this work were similar to the strains which were used in Part I and have shown no significant change in their resistance to 5-aminoacridine. However, in view of the tendency nowadays to explain differences in results from various laboratories in terms of strain variation, exhaustive experiments comparing the sensitivity of more recently isolated species with those labelled "stock strains" were performed. For this puprose ten strains of each type-species from widely differing sources were examined.

TABLE II.—*The Variation in Susceptibility of Recently Isolated Species. Highest Dilutions Completely Preventing Visible Growth in 48 Hours at 37° C.*

Medium: 10 per cent serum broth, pH 7·2–7·4.

Number of strains examined.	Species.	Number of strains inhibited by 5-aminonacridine at a dilution of 1 in—					
		20,000.	40,000.	80,000.	160,000.	320,000.	640,000.
10	*Clostridium welchii*	10	10	9	8	1	0
	Stock strain	—	—	—	+	—	—
10	*Streptococcus pyogenes A*	10	10	8	2	0	0
	Stock strain	—	—	—	+	—	—
10	*Staphylococcus aureus*	10	7	0	0	0	0
	Stock strain	—	+	—	—	—	—
10	*Bacterium coli*	10	9	1	0	0	0
	Stock strain	—	+	—	—	—	—
10	*Proteus* (species not identified)	10	10	0	0	0	0
	Stock strain	—	+	—	—	—	—

+ indicates inhibitory dilution for stock strain.

It will be noted, Table II, that the inhibition of growth by a typical derivative, 5-aminoacridine, is fairly constant for *Clostridium welchii* and *Streptococcus pyogenes*, and remarkably constant for strains of *Staphylococcus aureus*, *Bacterium coli* and *Proteus*.

Inoculum.

The size of inoculum used in bacteriostatic tests is subject to wide variation, and the choice of small inocula (e.g. 1000–2000 organisms) is important with some drugs (sulphonamides), but unimportant in the case of the aminoacridines. Experience in this study has shown that wide variations in number of organisms inoculated per

tube, namely, from 50 up to 5 million organisms, do not affect the bacteriostatic end-point after 48 hours' incubation (Table III).

TABLE III.—*The Effect of Size of Inoculum on the Bacteriostatic End-point.*

Organism : *Staphylococcus aureus.*

Medium : 10 per cent serum broth, pH 7·2–7·4.

Number of organisms inoculated per tube (based on plate counts).	Time of incubation in hours.	Dilutions of 5-aminoacridine.			
		3.	4.	5.	6.
$4{\cdot}5 \times 10^7$	24	±	±	+	+
	48	+	+	+	+
$4{\cdot}5 \times 10^6$	24	−	−	+	+
	48	−	−	+	+
$4{\cdot}5 \times 10^5$	24	−	−	+	+
	48	−	−	+	+
$4{\cdot}5 \times 10^4$	24	−	−	+	+
	48	−	−	+	+
$4{\cdot}5 \times 10^3$	24	−	−	−	+
	48	−	−	+	+
$4{\cdot}5 \times 10^2$	24	−	−	−	+
	48	−	−	+	+
$4{\cdot}5 \times 10^1$	24	−	−	−	+
	48	−	−	+	+
4	24	−	−	−	+
	48	−	−	±	+

− Signifies no growth ; ± signifies slight growth ; + signifies growth.

In order to obtain results of potential clinical significance care has been taken to favour the growth of the organism by adhering to the use of a " heavy " inoculum throughout this work (Garrod, 1935). This was standardized as 0·03 ml. (delivered as one drop from a Pasteur pipette) of a 24-hour culture to 2·5 ml. of medium, and contained a million or more organisms.

Method.

Geometrical dilutions of each derivative were made in 25 ml. quantities of serum broth commencing at 1 in 5000 concentration. The range covered was such that the two highest dilutions showed organismal growth. *That dilution completely preventing visible growth after 48 hours' incubation at 37° C. was taken as the end-point of bacteriostasis, and to this dilution a code number was assigned according to the following key:*

Key to Dilutions to be Used for Tables III, IV, V, VI, VII, IX and X.

0	signifies	growth	at	1 in	5000 of base.
1	signifies	inhibition of growth	at	1 in	5,000.
2	,,	,,	,,	1 ,,	10,000.
3	,,	,,	,,	1 ,,	20,000.
4	,,	,,	,,	1 ,,	40,000.
5	,,	,,	,,	1 ,,	80,000.
6	,,	,,	,,	1 ,,	160,000.
7	,,	,,	,,	1 ,,	320,000.
8	,,	,,	,,	1 ,,	640,000.
9	,,	,,	,,	1 ,,	1,280,000.
10	,,	,,	,,	1 ,,	2,560,000.

All tests have been done in duplicate and, with important compounds, repeated again in duplicate.

Interpretation of results.

The " bacteriostatic index " or B.I. of a substance refers to the sum of the code numbers of the inhibitory dilutions for each organism. A difference of 5 units in the bacteriostatic index represents on the average a twofold change in concentration necessary to effect bacteriostasis. An increase of 5 units is considered to be suggestive of heightened antibacterial action, whereas a figure greater than 5 units is quite significant. We have come to regard B.I. value of less than 10 as indicative of poor antibacterial action, a B.I. of 15 suggesting moderate activity, whilst a derivative with a well distributed B.I. above 20 is considered to have marked activity. These arbitrary figures have been based on the bacteriostatic indices of aminoacridines of proven clinical usefulness, viz. proflavine, euflavine (acriflavine), 2 : 7-diaminoacridine, 5-aminoacridine and " Rivanol." It is not implied, however, that a drug, to be of therapeutic significance, must possess a B.I. greater than 20, for there are many other factors to be considered before making this decision, particularly histo-pathological data. In regard to comparison of scores for individual organisms, a difference of 2 units, equal to a fourfold difference in concentration, is considered significant. However, compounds that score 2 or less for an organism cannot be accurately compared, since slight precipitation occurs in some cases at these high concentrations (i.e. 1 in 10,000). We have not recorded where such precipitation occurs in this series, since the general conclusions are unaffected by this factor.

Classification of material.

For convenience in presenting the bacteriological data pertaining to the many derivatives of acridine, the material has been classified in Tables IV–VII into four categories based mainly on the degree of ionization (or basicity) of each acridine compound. (It is appropriate to point out here that highly basic acridines are almost completely ionized in solution to give kations.)

RESULTS.

Highly Ionized Acridine Compounds.

The compounds falling within this group have a basic strength equal to or greater than 7·8 (expressed in pK_a units) which, in terms of ionization, is equivalent to 75 per cent or more of the base in the kationic form at pH 7·3 at a temperature of 20° C. This corresponds to 60 per cent ionization at 37° C. (Hall and Sprinkle, 1932). O the 50 compounds set out in Table IV, it will be noted that all except Nos. 23, 24 and 50 have a B.I. equal to or greater than 15, and hence may be regarded as active drugs. It will also be noted that although the pK_a values vary quite considerably (from 7·5 to 12·0), this variation is not associated with proportionate variations in antibacterial action, showing that once sufficient ionization has been achieved, further increases in pK_a value do not influence the activity (Fig. 3).

Within this series of adequately ionized derivatives some significant variations in antibacterial activity are to be seen, and it appears that factors, other than ionization, must be concerned in contributing towards antibacterial action. Nevertheless, these factors are of secondary importance, and manifest themselves only when the prime factor, ionization, is accomplished. The significance of substituent groups in this connection will be discussed shortly.

TABLE IV.—*Compounds Sufficiently Ionized to Possess Marked Antibacterial Activity. Highest Dilutions Completely Preventing Visible Growth in 48 Hours at 37° C.*

Medium : 10 per cent serum broth, pH 7·2–7·4.

Number.	Substance.	Organisms.					Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pK_a in water at 20° C.	Per cent. ionized (kation) at 20° C., pH 7·3.
		Cl. welchii.	*Strep. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*			
	Monoaminoacridines.								
1	2-Aminoacridine	6	5	3	4	3	21	8·2	88
2	5-Aminoacridine	7	6	4	4	4	25	9·9	100
	Diaminoacridines.								
3	2 : 5-Diaminoacridine	7	6	2	2	0	17	11·4	100
4	3 : 5-Diaminoacridine	7	6	2	2	1	18	10·5	100
5	2 : 6-Diaminoacridine	7	7	4	4	4	26	9·2	99
6	2 : 7-Diaminoacridine (*"acramine red"*)	7	6	4	5	4	26	8·2	88
7	2 : 8-Diaminoacridine (proflavine)	7	6	4	3	2	22	10·0	100
	Substituted 5-Aminoacridines.								
8	5-Amino-1-methylacridine	7	7	5	4	4	27	10·2	100
9	5-Amino-2-methylacridine	6	6	4	3	2	21	10·2	100
10	5-Amino-3-methylacridine	7	6	4	4	2	23	10·0	100
11	5-Amino-4 methylacridine	6	6	4	4	4	24	10·0	100
12	5-Amino-1 : 3-dimethylacridine	8	7	5	3	1	24	10·5	100
13	5-Amino-1 : 9-dimethylacridine	8	7	5	4	3	27	9·3	99
13*a*	5-Amino-1-ethylacridine	7	6	4	3	2	22	10·2	100
14	5-Amino-1-methoxyacridine	7	6	4	3	2	22	9·9	100
15	5-Amino-2-methoxyacridine	7	6	5	4	3	25	10·1	100
16	5-Amino-3-methoxyacridine	7	7	5	4	2	25	9·6	100
17	5-Amino-4-methoxyacridine	7	6	4	3	2	22	10·2	100
18	5-Amino-1-chloroacridine	6	5	4	3	2	20	8·3	91
19	5-Amino-2-chloroacridine	7	6	5	5	3	26	9·0	98
20	5-Amino-3-chloroacridine	7	6	5	4	4	26	8·8	97
21	5-Amino-4-chloroacridine	6	6	4	4	2	22	8·4	93
22	5-Amino-1-hydroxyacridine	5	5	3	3	3	19	8·3	91
23	5-Amino-2-hydroxyacridine	2	4	0	0	0	6	7·7	71
24	5-Amino-3-hydroxyacridine	4	6	2	0	0	12	9·1	98
25	[5-Amino-4-hydroxyacridine]*	3	3	1	0	0	[7]*	[6·5]*	[11]*
26	5-Amino-1-nitroacridine	6	7	4	4	3	24	7·8	76
27	5-Amino-2-nitroacridine	6	10	6	5	3	30	7·8	76
28	5-Amino-3-nitroacridine	7	6	5	4	2	24	7·9	80
29	5-Amino-4-nitroacridine	7	8	6	4	2	27	7·5	61
30	5-Amino-1-phenylacridine	5	5	3	2	0	15	9·2	98
31	5-Amino-3-phenylacridine	8	7	6	3	0	24	9·7	100
32	5-Amino-3-cyanoacridine	7	5	4	4	3	23	8·0	83
33	5-Amino-2-nitro-7-ethoxyacridine	7	8	4	0	0	19	7·5	60
34	5-Amino-2-chloro-7-methoxyacridine	8	6	5	2	0	21	8·6	95
	5-Amino-10-methylacridinium hydroxide (as chloride)	6	6	4	4	3	23	10·6	100
35	Ditto (as bromide)	7	6	4	4	3	24	10·6	100
	Ditto (as anhydro-base ; 5-imino-10-methylacridane)	7	7	5	5	4	28	10·6	100
36	5-Methylaminoacridine	7	6	4	2	3	22	10·1	100
37	5-(β-Hydroxyethyl)-aminoacridine	5	5	2	2	1	15	9·3	99
38	5-Amino-3-carbomethoxyacridine	7	6	5	2	0	20	8·5	94
39	5-Aminoacridine-3-carboxamide	3	5	2	3	2	15	8·7	96

TABLE IV (*continued*).

Number.	Substance.	Organisms. *Cl. welchii.*	*Strep. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*	Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pK_a in water at 20° C.	Per cent. ionized (kation) at 20° C., pH 7·3.	Per cent. ionized (kation) at 20° C., pH 7·3.
	Substituted Diaminoacridines.									
40	2 : 8-Diamino-1 : 9-dimethylacridine	9	8	6	4	3	30	9·0	98	88
41	2 : 8-Diamino-3 : 7-dimethylacridine (acridine yellow)	8	6	4	3	0	21	10·3	100	100
42	2 : 8-Diamino-4 : 6-dimethylacridine	8	8	2	4	0	22	10·4	100	100
43	2 : 8-Diamino-3 : 7-dichloroacridine	8	6	4	5	0	23	8·2	88	99
44	2 : 8-Diamino-10-methylacridinium hydroxide (as chloride) (purified euflavine)	7	8	3	3	1	22	12	100	100
45	2 : 8-*bis*-dimethylaminoacridine (acridine orange)	5	6	4	2	0	17	10·8	100	100
46	2 : 5-Diamino-7-ethoxyacridine ("*Rivanol*")	6	7	4	3	0	20	11·5	100	100
47	2-Dimethylamino-7-aminoacridine	6	5	3	1	0	15	8·5	94	100
	Miscellaneous Acridines.									
48	2-Amino-10-methyl-acridinium hydroxide (as bromide)	6	7	1	5	0	19	12	100	100
49	3-Amino-5 : 10-dimethylacridinium hydroxide (as bromide)	5	5	3	2	0	15	10	100	100
50	Ω-Amino-3-methylacridine	3	2	0	0	0	5	9·2	98	99
51	Ω-Amino-5-ethylacridine	6	6	2	3	2	19	9·1	98	100

* Included here for comparison with other isomerides.

From the purely bacteriological point of view a striking feature in this and all other Tables is that the order of species sensitivity is almost invariable. It can be concluded that *Clostridium welchii* is the most sensitive organism of our series, followed by *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacterium coli* and *Proteus* in order of increasing resistance. In regard to *Clostridium welchii* it is worth mentioning that some poorly ionized acridine derivatives (Table V) have a decided action on this organism. This greater sensitivity may be due partly to lack of shunt respiratory enzyme systems, since it is known that oxidation by cytochrome-lacking bacteria (strict anaerobes) proceeds through more restricted channels (flavine nucleotides) than oxidations by cytochrome-containing bacteria (facultative anaerobes).

Poorly Ionized Acridine Compounds.

It will be seen that none of the 33 compounds (Table V, Nos. 52 to 84) possess marked antibacterial properties, and only three (Nos. 65, 77 and 78) possess even moderate activity. These findings, taken in conjunction with the pK_a values, all of which are below 7·8, lead to the conclusion that lack of significant activity is directly related to lack of sufficient ionization at pH 7·3.

TABLE V.—*Compounds Insufficiently Ionized to Possess Marked Antibacterial Activity. Highest Dilutions Completely Preventing Visible Growth in 48 Hours at 37° C.*

Medium : 10 per cent serum broth, pH 7·2–7·4.

Number.	Substance.	Organisms.					Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pK_a in water at 20° C.	Percentage ionized (kation) at 20° C., pH 7·3.
		Cl. welchii.	*Strep. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*			
52	Acridine	3	1	1	0	1	6	4·8	0·3
	Substituted acridines.								
53	1-Methylacridine	2	0	0	0	0	2	4·5	0·2
54	3-Methylacridine	0	0	0	0	0	0	5·1	0·6
55	5-Methylacridine	3	0	0	0	0	3	5·2	0·7
56	1 : 3 : 4 : 6 : 7 : 9-Hexamethylacridine	0	0	0	0	0	0	3·5	<0·01
57	1-Methoxyacridine	3	0	0	0	0	3	4·7	0·3
58	3-Methoxyacridine	3	2	1	0	0	6	4·8	0·3
59	2 : 8-Dimethoxyacridine	5	4	0	0	0	9	6·2	7·4
60	1-Acetamidoacridine	2	1	0	0	0	3	3·0	<0·01
61	2-Acetamidoacridine	2	2	0	0	0	4	5·6	2·0
62	3-Acetamidoacridine	4	2	1	1	1	9	4·7	0·3
63	4-Acetamidoacridine	0	0	0	0	0	0	4·4	0·1
64	5-Acetamidoacridine	0	0	0	0	0	0	4·3	0·1
65	1-Hydroxyacridine	5	4	3	0	0	12	4·7	0·3
66	2-Hydroxyacridine	3	2	1	0	0	6	5·4	1·2
67	3-Hydroxyacridine	0	0	0	0	0	0	4·7	0·3
68	4-Hydroxyacridine	0	0	0	0	0	0	5·0	0·5
69	5-Hydroxyacridine (acridone)	0	0	0	0	0	0	<2	<0·01
	Monoaminoacridines.								
70	1-Aminoacridine	2	1	1	0	0	4	4·2	0·1
71	3-Aminoacridine	5	2	1	0	0	8	5·8	3·0
72	3-Aminoacridane	2	2	1	0	0	5	5·2	0·7
73	4-Aminoacridine	5	2	1	1	0	9	6·2	7·4
	Substituted Aminoacridines.								
74	3-Amino-5-methylacridine	5	3	2	0	0	10	6·1	6·0
75	3-Amino-8-chloroacridine	0	0	0	0	0	0	4·5	0·2
76	2-Amino-5-chloroacridine	1	0	0	0	0	1	6·5	13
77	2-Amino-7-chloroacridine	5	4	3	1	0	13	7·0	33
78	2-Amino-8-chloroacridine	4	4	3	2	0	13	7·3	50
	Diaminoacridines.								
79	2 : 9-Diaminoacridine	4	3	0	2	0	9	7·2	44
79*a*	1 : 9-Diaminoacridine	0	0	0	0	0	0	4·3	0·1
80	3 : 7-Diaminoacridine	5	3	1	0	0	9	6·2	7·4
	Miscellaneous Acridines.								
81	10-Methylacridinium hydroxide (as bromide	0	0	0	0	0	0	(unstable)	?
82	3-Amino-10-Methylacridinium hydroxide (as bromide)	1	2	0	0	0	3	„	?
83	4-Amino-10-Methylacrinidium hydroxide (as bromide)	3	3	2	0	0	8	„	?
84	*p*-Amino-5-styrylacridine	0	0	0	0	0	0	5·6	2·0

Correlation of Ionization with Antibacterial Activity.

The connection between ionization and activity is well illustrated when we analyse the effect of substituents attached to the acridine nucleus. It will be seen that substituents influence antibacterial activity in the general direction predictable by changes in ionization of the new derivative.

Amino-derivatives.

The mono-aminoacridines (Nos. 1, 2, 70, 71 and 73) have been previously described, and it has been shown that the insertion of an amino group into the 2- or 5-position induces a considerable increase in ionization, and a corresponding increase in antibacterial activity (Rubbo, Albert and Maxwell, 1942). The insertion of an amino group in the 1-, 3- or 4-position has little influence on ionization, and for this reason the activity of these derivatives is of the same order as that of acridine itself (No. 52). In the same way, it now transpires that the activity of the diaminoacridines depends not so much on the position of the amino groups (*cf.* Albert, Francis, Garrod and Linnell, 1938) as on the ionization that they engender. For example, the well ionized diaminoacridines (Nos. 3–7) are highly active, whilst the poorly ionized diaminoacridines (Nos. 79*a*, 79 and 80) have only poor activity in spite of the fact that one of them (2 : 9-diaminoacridine) possesses an amino group in the 2-position.

Chloro-derivatives.

The insertion of chlorine, which always depresses the pK_a value by approximately one unit, will, or will not, depress activity, depending entirely upon the final pK_a of the new derivative. This is well shown in the chloroderivatives of 2- and 5- aminoacridines, the former being relatively inactive when compared with 2-aminoacridine (*cf.* No. 1 with Nos. 76–78), whilst the latter are quite active when compared with 5-aminoacridine (*cf.* No. 2 with Nos. 18–21). In the former instance, the pK_a of 2-aminoacridine, which is near the critical value of 7·8, could not withstand further reduction of 1 unit and still retain adequate ionization, whereas after a depression of 1 unit in the pK_a of 5-aminoacridine, a fully ionized derivative still remains. Berry (1941) examined three chloroaminoacridines common to the present series, and whilst there is good general agreement between the two sets of results, he found 2-amino-8-chloroacridine far less active than we did. Whilst we are at a loss to explain this difference, our results are consistent with the general principles of ionization.

Nitro-derivatives.

The nitro-substituted derivatives of 5-aminoacridine (Table IV, Nos. 26–29 and 33) are of extreme interest, since the nitro group is a base-weakening group and, in spite of the poor ionization that it sometimes engenders, the amines which have been substituted in this way have actually become more active. This exaltation is most pronounced when the nitro-group occupies the 2-position, and is so significant that the 5-amino-2-nitroacridines rank amongst the most active acridines.

A number of antibacterial nitro-compounds are known, the simplest of them being *p*-nitrobenzoic acid, and it must be conceded that nitro-compounds form a distinct class of antiseptics which function, in the first place, through an oxidizing action. The reduction of nitro-compounds in neutral media, which takes place very readily, usually leads to hydroxylamino derivatives, which in the present instance would restore the basicity to at least the required figure of 7·8. In addition, hydroxylamino compounds

have antibacterial properties of their own. Green and Bielschowsky (1942) investigated the antibacterial action of *p*-nitrobenzene sulphonamide and found it was probably reduced to *p*-hydroxylaminobenzene sulphonamide, but not to *p*-aminobenzene sulphonamide.

In expectation that a change in the intensity and colour of fluorescence would accompany the interaction of bacteria with these nitroaminoacridines, indicating reduction to hydroxylamino derivatives, the following experiments were performed:

Serial dilutions of 2 : 5-diaminoacridine, 3 : 5-diaminoacridine, 2-nitro-5-aminoacridine and 3-nitro-5-aminoacridine were made in 10 per cent. serum broth covering a range from 1 in 20,000 to 1 in 640,000, and half the tubes were inoculated with *Clostridium welchii*, *Staphylococcus aureus* and *Bacterium coli*. The two sets of tubes were incubated for 48 hours at 37° C. and then examined for any change in fluorescence or colour intensity, using a Hanovia ultra-violet lamp fitted with a Woods glass filter. In one case, namely, in the higher dilutions of 2-nitro-5-aminoacridine in which *Bacterium coli* grew, the fluorescence changed from a moderately bluish one to a dazzling golden-yellow colour similar to that of 2 : 5-diaminoacridine. As no other examples of this change were observed it could not be concluded that nitro-compounds are necessarily reduced to the corresponding amine or hydroxylamine, hence the source of their great activity is not yet demonstrated.

Methyl derivatives.

The introduction of methyl groups into highly ionized acridines, particularly in the 1-position, somewhat enhances the antibacterial action. This effect is seen by comparing proflavine (No. 7) and 1 : 9-dimethyl-proflavine (No. 40), whose respective B.I.'s are 22 and 30. It is believed that specific absorption on bacterial enzymes has been heightened by the insertion of a methyl group contiguous to the ring-nitrogen, as in the above example.

Hydroxy derivatives.

Of the five isomeric mono-hydroxyacridines (Table V, Nos. 65–69) compared for the first time, it will be seen that all are poorly ionized and that only the 1-isomeride has any noteworthy activity. We are inclined to attribute this to a chelating action between the hydroxy group and the ring nitrogen whereby divalent metals essential in cellular metabolism could be immobilized. Zentmeyer (1944) has shown that this phenomenon underlies the activity of the analogously constituted 8-hydroxyquinoline.

The hydroxy derivatives of 5-aminoacridine (Nos. 22–25) show an activity somewhat less than would be predicted from their pK_1 values. For instance, the 2- and 3-derivatives (Nos. 23 and 24) are ionized to the extent of 71 and 98 per cent respectively, and yet their B.I. values are only 6 and 12. This may be due to adsorption elsewhere than on the vital acidic groups normally combining with aminoacridines, and the strong hydrogen-bonding effects of —OH groups makes this a likely explanation. Once again, the 1-hydroxy-isomeride achieves the highest score. In 5-(β-hydroxyethyl)aminoacridine (No. 37) a further example of partial deactivation by a hydroxy group is seen.

Miscellaneous derivatives.

In the majority of instances the introduction of methyl, phenyl, methoxy and ethoxy groups has only a slight effect on the ionization and hence does not effect antibacterial action (viz. Table IV, Nos. 8–17, 30, 31, 33, 34 and 46). Finally we come to consider those acridine compounds grouped in Table V, which do not possess amino

substituents. In all cases the pK_a is of much the same order as acridine itself, and hence ionization and antibacterial activity are very low.

Correlation of Ionization with Molecular Structure.

Since the findings described in Tables IV and V suggest a positive connection between ionization and antibacterial activity, it will be convenient at this stage to describe methods by which acridine, which is poorly ionized, may be endowed with a basicity sufficiently high to effect adequate ionization of the new derivative at pH 7·3.

There are three methods by which the pK_a value of acridine may be exalted to the required value of pK_a 7·8 or more. They are: (1) By the insertion of one or more amino groups in the 2- or 5-position; (2) by insulation of an amino group from the base-weakening effect of the nucleus. This gives, in effect, a basic side-chain; (3) by quaternization of the ring nitrogen.

As regards method 1, the insertion of an amino group in the 2- or 5-position leads to a very considerable increase in basicity of the new amino derivative, when compared with acridine or with the 1-, 3- or 4-aminoacridines. This increase in basicity is due to extra resonance in the ion, which, by virtue of the geometry of the molecule, can occur only when the amino group is in the 2- or 5-position (Albert and Goldacre, 1943). It must be appreciated that a distinction is drawn between resonance, which acridine possesses, and extra-ionic resonance, which it does not (Branch and Calvin, 1941).

As already noted, the 2- and 5-aminoacridines (Table IV, Nos. 1 and 2) and their derivatives (Table IV) have marked bacteriostatic capacities, and our main problem at the moment is to decide to what extent ionization or ionic resonance or a combination of both contribute towards activity. This point is peculiarly difficult to resolve chemically because those aminoacridines which are highly basic, and hence highly ionized, usually have ionic resonance, whereas those which are weakly ionized are usually without ionic resonance.

Relative Importance of Ionization and Ionic Resonance.

Whilst all derivatives of 2- and of 5-aminoacridines possess ionic resonance, fortunately not all are fully ionized as kations, and it is with these derivatives that the opportunity for assessing the importance of ionic resonance *per se* presents itself.

The chloro-derivatives of 2-aminoacridine, whose relative inactivity has been discussed, may again be quoted here as examples of acridines possessing ionic resonance but lacking in adequate ionization.

The insertion of acidic groups into the nuclei of 2- and 5-aminoacridines produces derivatives whose kationic ionization has been replaced by zwitterionic ionization due to internal salt formation. This manoeuvre makes it possible to demonstrate that kationic ionization, distinct from ionic resonance, is essential for antibacterial action in the acridine series.

For example, 2-aminoacridine-7-sulphonic acid (Table VI, No. 90) has an absorption spectrum practically identical with that of 2-aminoacridine (Goldacre, unpublished) and thus has the same ionic resonance, but 65 per cent is in the zwitterionic form and 35 per cent. in anionic form. This substance is completely devoid of antibacterial action (B.I. = 1). On the other hand, the acidic ionization of this compound may be eliminated by forming the amide (Table VI, No. 91) which, by virtue of its 44 per cent ionization as a base, now possesses an antibacterial activity (B.I. = 8) predictable in terms of kationic ionization. This compound is of interest, for it throws some light on the difficulty of trying to combine two antibacterial actions (sulphonamide and aminoacridine) in the one molecule, when these actions depend on entirely different

TABLE VI.—*The Suppression of Antibacterial Activity Owing to Zwitterion Structure. Highest Dilutions Completely Preventing Visible Growth in 48 Hours at 37° C.*

Medium : 10 per cent serum broth, pH 7·2–7·4.

Number.	Substance.	Organisms.					Bacteriostatic index (sum total of code numbers of inhibitory dilutions).	pK_a values in water at 20° C.		Percentage ionized at 20° C. pH 7·3.		
		Cl. welchii.	*Strep. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*		Base.	Acid.	As kation.	As anion.	As zwitterion.
(52)	*Acridine* (for comparison)	3	1	1	0	1	6	4·8	None	0·3	*Nil*	*Nil*
85	Acridine-3-sulphonic acid	0	0	0	0	0	0	4·7	*ca.* 1·5	0·0	99·5	0·5
86	Acridine-1-carboxylic acid	0	0	0	0	0	0	8·0	*ca.* 2	0·0	17	83
87	Acridine-3-carboxylic acid	0	0	0	0	0	0	5·2	*ca.* 2	0·0	99·1	0·9
88	Acridine-5-carboxylic acid	0	0	0	0	0	0	5·0	*ca.* 2	0·0	99·3	0·7
89	Methyl ester of last named	0	0	0	0	0	0	3·5	None	0·0	*Nil*	*Nil*
(1)	2-*Aminoacridine* (for comparison)	6	5	3	4	3	21	8·2	,,	88	,,	,,
90	2-Aminoacridine-7-sulphonic acid	1	0	0	0	0	1	7·6	*ca.* 2	0	34	66
91	2-Aminoacridine-7-sulphonamide	4	2	0	2	0	8	7·2	*ca.* 11	44	0·05	0·02
92	2-Aminoacridine-7-carboxylic acid	0	0	0	0	0	0	8·0	*ca.* 2	0	16	84
93	Methyl ester of last named	1	0	0	0	0	1	6·9	None	29	*Nil*	*Nil*
(2)	5-*Aminoacridine* (for comparison)	7	6	4	4	4	25	9·9	,,	100	,,	,,
94	5-Aminoacridine-3-carboxylic acid	0	0	0	0	0	0	9·9	3·0	0	0·2	99·8
(38)	Methyl ester of last named	7	6	5	2	0	20	8·5	None	94	*Nil*	*Nil*
(39)	5-Aminoacridine-3-carboxamide	3	5	2	3	2	15	8·7	,,	96	,,	,,
95	5-Aminoacridine-1-carboxylic acid	0	0	0	0	0	0	13·0	2·7	0·0	0·0	100

mechanisms, one acting as an anionic drug, the other as a kationic. The —COOH, —$COOCH_3$ and —$CONH_2$ derivatives of 5-aminoacridine (Nos. 94, 38 and 39) are of extraordinary interest in that we have :

5-aminoacridine : normal kationic ionization . . .	active (No. 2).
—COOH derivative : complete internal neutralization .	inactive (No. 94).
—$COOCH_3$ derivative : normal kationic ionization . .	active (No. 38).
—$CONH_2$ derivative : normal kationic ionization . .	active (No. 39).

As all these derivatives possess ionic resonance structures, it becomes obvious that ionic resonance without sufficient kationic ionization is of no value in determining antibacterial action. Furthermore these facts demonstrate that activity is dependent entirely on kationic (as opposed to zwitterionic or anionic) ionization.

Quite a different way of producing well ionized acridine compounds without introducing ionic resonance is exemplified by Ω-amino-3-methylacridine and Ω-amino-5-ethylacridine (Table IV, Nos. 50 and 51), which were prepared along the lines of method 2 above, namely, by insertion of a basic side chain. In this case the amino group is not directly attached to the nucleus, so that the free bases are almost as strong as the aliphatic amines, methylamine and ethylamine, from which they are derived. Chemically these compounds have adequate ionization (pK_a 9·0), but are lacking in ionic resonance. The figures obtained for Ω-amino-5-ethylacridine (No. 51) demonstrate that a fairly high antibacterial activity can be obtained in the absence of ionic resonance provided that ionization is sufficient.

The other example, Ω-amino-3-methylacridine (No. 50), like other Ω-aminomethyl compounds of the Mannich-base type, is chemically somewhat unstable. However, it was not entirely decomposed under the conditions of the tests, so that this may not be the sole explanation of its low score. These omega compounds, although difficult of access, would repay further study.

A third possibility remained of introducing adequate ionization into acridines without invoking either ionic resonance or a basic side-chain. This can be accomplished in selected cases by quaternization of the ring nitrogen (method 3 above).

In attempting to evolve a suitable example we were confronted with a problem that has been quite insufficiently discussed in chemical literature, namely, the variable, but often considerable instability of the quaternary compounds of heterocyclic bases. Our experience was that when 3- and 4-amino-10-methylacridinium bromides were shaken in phosphate buffer with air in a Warburg apparatus, oxygen was readily taken up and the corresponding acridones, which are quite unionized and biologically inactive, were obtained (see Albert and Ritchie, 1943). 10-methylacridinium bromide is known to behave similarly (Sidgwick, 1937).

Because of the instability of these substances which, in their intact state, are highly ionized structures lacking ionic resonance, it was difficult to decide whether their biological inactivity was due to the absence of ionic resonance or to dehydrogenation to the non-basic acridones. Their activities have been examined (Nos. 81, 82, 83), and it will be noted that none possesses strong antibacterial properties (B.I. values from 0 to 8).

However, this difficulty was overcome by stabilizing the quaternary compound by inserting a methyl group in the 5-position of 3-amino-10-methylacridinium bromide (Table IV, No. 49). This compound, which is incapable of oxidation to an acridone and did not absorb oxygen in the Warburg apparatus, showed significant activity (B.I. = 15) when compared with other quaternary compounds, but was not as active as one would expect if ionization were the sole determining factor. It is, therefore, unwise in the present state of our knowledge to disregard the coupling of ionic resonance

TABLE VII.—*Partial Suppression of Antibacterial Activity by Dimensional Factors. Highest Dilutions Completely Preventing Visible Growth in 48 Hours at 37° C.*

Medium : 10 per cent serum broth, pH 7·2–7·4.

Number.	Substance.	Organisms.					Bacteriostatic Index (sum total of code numbers of inhibitory dilutions).	Molecular weight.	Bacteriostatic index corrected for molecular weight.	pK_a in water at 20° C.	Percentage ionized (kation) at 20° C., pH 7·3.
		Cl. welchii.	*Strep. pyogenes.*	*Staph. aureus.*	*B. coli.*	*Proteus.*					
2	5-Aminoacridine (for comparison)	7	6	4	4	4	25	194	25	9·9	100
96	5-Butylaminoacridine	6	5	2	0	0	13	250	14	9·8	100
97	5-*Cyclo*hexylaminoacridine	6	5	3	0	0	14	276	15	9·2	99
98	5-Heptylaminoacridine	4	4	4	1	0	13	292	15	9·7	100
99	5-Dodecylaminoacridine	2	5	4	0	0	11	362	14	9·5	99
100	5-Hexadecylaminoacridine	3	3	2	0	0	8	418	11	9·3	99
101	5-Amino-6 : 7 : 8 : 9-tetrahydroacridine	1	1	0	0	0	2	198	2	9·9	100
102	2-Amino-5-*p*-aminophenylacridine (phosphine)	6	4	3	1	0	14	286	16	8·2	88
103	2 : 8-Diamino-3 : 7-dimethyl-5-phenylacridine (benzoflavine)	5	5	5	1	0	16	313	20	9·6	100
104	2-Chloro-7-methoxy-5-(1′-methyl-4′-diethylaminobutyl)aminoacridine ("*Atebrin*")	4	3	0	0	0	7	400	9	10·2	100
105	2 : 8 : 2′ : 8′-Tetra-amino-5 : 10-d-hydrodiacridyl-5 : 5′ ether	1	3	2	0	0	6	434	9	*ca.* 10	100
106	N : N′-*bis*(5-acridyl)ethylene diamine	5	3	1	0	0	9	414	12	9·0	98

and ionization with antiseptic activity of the aminoacridines; the possibility that both may play a direct, if interdependent, role in antibacterial activity is a real one. A number of similar compounds were prepared by Sharp, Sutherland and Wilson (1943) and have been reported on by Browning and Calver (1943), whose results confirm the above conclusions.

In the course of this study a few substances have been found which possess both of the physico-chemical attributes that we have been discussing but which, nevertheless, fail to reach a significant bacteriostatic index. These exceptions, which are both interesting and instructive, will be discussed at once as they throw further light on the relationship between structure and action in this series.

Relative Importance of the "Dimensional Factor."

We use the term " dimensional factor " to describe the suppression of antibacterial activity of a derivative (when compared with the parent molecule), by an apparent structural interference with the union of drug and the bacterial enzymes. Alles (1944) coined the term to describe similar effects in cholinergic drugs. As will be seen in the several examples shown in Table VII, the most obvious molecular changes have been either to increase the molecular dimension beyond a limiting value for the direction investigated or to destroy the planarity of the original molecule. These alterations are invariably associated with a significant loss of activity which, with the evidence available, can most reasonably be attributed to such a limiting dimensional factor. This, in turn, throws some light on the dimensions of the enzyme surface itself.

One of the most interesting examples of the dystherapeutic effects of the dimensional factor is seen in 5-amino-6 : 7 : 8 : 9-tetrahydroacridine (No. 101) (see also Fig. 1), which has the necessary ionization and ionic resonance associated with marked activity, but is completely devoid of antibacterial action (B.I. = 2). Although this compound differs from 5-aminoacridine (B.I. = 25) by only four hydrogen atoms, this seemingly small change in the chemical formula (see Fig. 1) has led to a considerable change in the steric picture. A completely conjugated molecule like 5-aminoacridine is virtually a two-dimensional structure in that all the atoms lie in the plane of the paper upon which the formula is drawn, whereas hydrogenation of one ring has the effect of buckling that ring. The result has been that perfect apposition with the adsorbing portion of the enzyme is hindered and consequently bacteriostasis is not realized (see " Discussion ").

In brief, the flat portion of the molecule of 5-amino-tetrahydroacridine has been reduced to the area of 4-aminoquinoline (see Fig. 1), which is also bacteriologically inactive.

An almost equally dystherapeutic effect may be obtained by doubling the size of the molecule as in N : N'-*bis*(5-acridyl)ethylenediamine (Table VII, No. 106), where two molecules of 5-methylaminoacridine (Table IV, No. 36) have been joined together by their methyl groups (see Fig. 1), thus reducing the B.I. from 22 to 9. Similarly with No. 105 (Table VII), where a molecule of proflavine has been coupled to one of dihydroproflavine by a simple ether linkage, thus reducing the B.I. from 22 to 6. Naturally, smaller additions to the molecule have smaller effects; the addition of a *p*-aminophenyl group to 2-aminoacridine, giving phosphine (Table VII, No. 102), has reduced the B.I. from 21 only to 14, and the addition of a phenyl group to acridine yellow (Table IV, No. 41) has reduced the B.I. from 21 only to 16 in benzoflavine (Table VII, No. 103). Here, as usual, the dimensional factor affects some bacterial types more than others.

The inactivity of "*Atebrin*" (Table VII, No. 104) compared to 5-amino-2-chloro-7-methoxyacridine, i.e. the same compound without the side-chain (Table IV, No. 34),

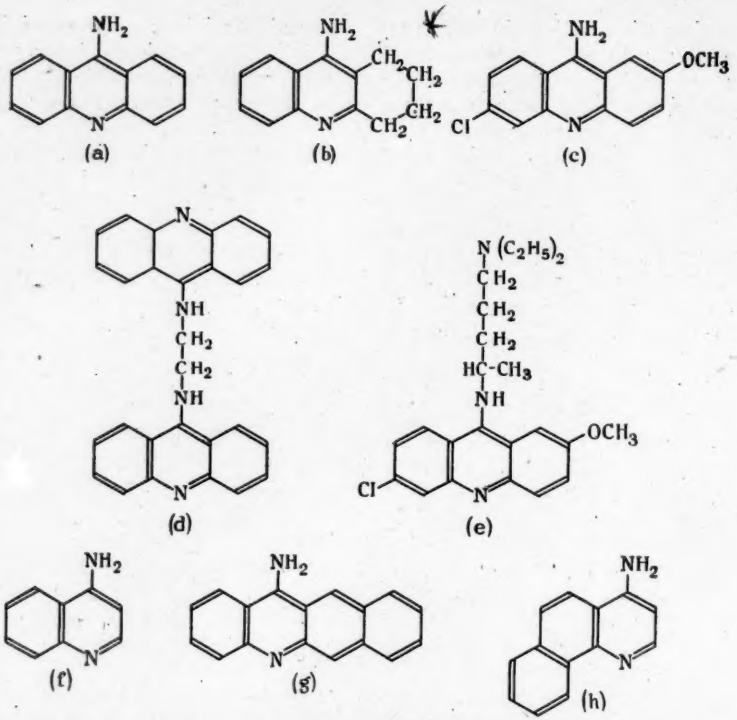

FIG. 1.—(*a*) 5-Aminoacridine (B.I. = 25). (*b*) 5-Amino-tetrahydroacridine (B.I. = 2). (*c*) 5-Amino-2-chloro-7-methoxyacridine (B.I. = 21). (*d*) N : N′-*bis* (5-acridyl)-ethylenediamine (B.I. = 8). (*e*) "*Atebrin*" (B.I. = 7). (*f*) 4-Aminoquinoline (B.I. = 0). (*g*) 5-Amino-2 : 3-benzacridine (B.I. = 26). (*h*) 4-Aminobenzo-h-quinoline (B.I. = 18).

is noteworthy, the B.I. falling from 21 to 7. These two compounds are illustrated in Fig. 1. Goetchius and Lawrence (1944) point out that the removal of the methyl group from "*Atebrin*," giving 2-chloro-7-methoxy-5 (4′-diethylaminobutyl)aminoacridine, somewhat increases its activity against Gram-positive organisms, and this may indicate some steric hindrance to the 5-amino group by the methyl of "*Atebrin*."

It should be noted that in none of these instances of dimensional interference has any fall of basicity occurred and ionization remains unimpaired. However, in many cases a substantial increase in molecular weight has taken place. The sixth column of Table VII enables a comparison to be made on a molecular rather than on a weight basis. However, the figures thus obtained do not change the conclusions already reached.

It is noteworthy that in Table VII all the extensions to the molecule have been made in the direction onwards from the 5-position. The 1- and 3-phenyl derivatives of 5-aminoacridine (Table IV, Nos. 30 and 31) and the new substance, 5-amino-2 : 3-benzacridine (Fig. 1), afford a glimpse of what extension in other directions may lead to.

The long-chain alkyl derivatives of 5-aminoacridine offer further examples of reduction in activity due to a limiting dimensional factor. It will be seen that the methyl compound (Table IV, No. 36, B.I. = 22) retains the activity of the parent

substance, but when the substitute, alkyl radical contains four or more carbon atoms the activity is significantly depressed (Table VII, Nos. 96–100). Actually, in these instances the depression appears to be the result of more than one factor, viz. lipophilic nature, but the dimensional effect associated with a bulky molecule cannot be disregarded.

Relative Importance of Lipophilic Nature.

The fact that a few highly ionized derivatives of 5-aminoacridine had a B.I. below 15 but marked lipophilic tendencies, made it desirable to examine a representative selection of acridines in order to determine the degree of lipophilic nature associated with varying degrees of activity. This property influences the proportion entering the lipoid phase of the cell, and consequently should provide some indication of the site of action of these drugs. A wide range of lipophilic tendencies is represented in this series which encompasses, between the extremes, a six thousand-fold increase in oil/water partition coefficient (Albert, Goldacre and Heymann, 1943). The results are depicted in Table VIII, the individual members being arranged in order of their increasing affinity for oil compared to their affinity for water.

TABLE VIII.—*Representative Compounds Illustrating that Marked Antibacterial Activity is Independent of Lipophilic Property or of Surface Activity at Serum-Protein/Water Interface.*

Table.	Number.	Substance.	Bacteriostatic index (sum total of code numbers of inhibitory dilutions).		Partition coefficient olive oil/water at 20° C. ± 5°) pH 7·1 (Albert, Goldacre and Heymann, 1943).	Percentage ionized at pH 7·3.
			10 per cent serum broth.	Broth.		
IV	44	2 : 8-Diamino-10-methyl-acridinium hydroxide (as chloride) (pure euflavine)	22	20	<0·2	100
IV	7	2 : 8-Diaminoacridine (proflavine)	22	21	0·7	100
IV	3	2 : 5-Diaminoacridine	17	16	1·0	100
IV	2	5-Aminoacridine	25	21	1·2	100
IV	1	2-Aminoacridine	21	21	5·2	88
IV	45	2 : 8-*bis*-Dimethylaminoacridine (acridine orange)	17	18	7·3	100
IV	36	5-Methylaminoacridine	22	20	12	100
VII	96	5-Butylaminoacridine	13	14	16	100
IV	46	2 : 5-Diamino-7-ethoxyacridine (Rivanol)	20	20	21	100
VII	104	*Atebrin*	7	5	31	100
V	73	4-Aminoacridine	9	8	55	7
VII	97	5-*Cyclo*hexylaminoacridine	14	14	77	99
V	71	3-Aminoacridine	8	9	90	3
V	52	Acridine	6	6	200	0·3
VII	98	5-Heptylaminoacridine	13	19	1000 (*ca.*)	100
V	70	1-Aminoacridine	4	4	1200	0·1
VII	99	5-Dodecylaminoacridine	11	14	too great to measure	99
VII	100	5-Hexadecylaminoacridine	8	6	too great to measure	99
Surface Active Kationic Compounds for Comparison.						
		Laurylamine	1	19	—	100
		Undecylamine	4	17	—	100
		Zephiran	13	21	—	100

It will be seen that the active compounds (i.e. B.I. more than 15) are markedly less soluble in oil than the majority of less active members. There is an easily recognizable negative correlation between activity and lipophilic nature, and it would appear, on this presumptive evidence, that a partition coefficient greater than 25 would prejudice against marked antibacterial action. However, it will be appreciated that the active members of Table VIII are highly ionized, whilst the more markedly lipophilic types are poorly ionized, with the exception of the alkyl derivatives of 5-aminoacridine. The latter owe their reduced bacteriostatic capacity at least partly to other factors, e.g. dimensional factors discussed above. The important conclusion may be drawn that the highest activity in the acridine series is consistent with a partition coefficient not far removed from unity, and this strongly suggests that the aminoacridines act in the watery phase of the bacterial cell.

Table VIII also shows the B.I. of each compound in 10 per cent. serum broth and in broth. The average B.I. for non-lipophilic members in each medium is 19, which permits the conclusion that acridines display little surface activity at the protein-water interface and hence are not inactivated by protein. This stands in very marked contrast to the other kationic antibacterials included for comparison, each of which is significantly depressed by serum protein, viz. " Zephiran " approximately threefold, undecylamine fivefold and laurylamine sixteenfold depressions.

Relative Importance of Reduction Potentials.

In view of the importance of oxidation-reduction potentials in cellular respiration and their determining influence on the transport of hydrogen through the agency of enzyme and co-enzyme systems, it seemed worth while to consider the reduction potentials of some representative acridines.

Breyer, Buchanan and Duewell (1944) reported that acridine and 1-, 3- and 4-aminoacridines have reduction potentials, when compared to the normal hydrogen electrode, of $-0{\cdot}31$, $-0{\cdot}39$, $-0{\cdot}37$ and $-0{\cdot}30$ volts at pH 7·3 respectively and that proflavine and 2- and 5-aminoacridines have values of $-0{\cdot}73$, $-0{\cdot}47$ and $-0{\cdot}92$ volts. In this restricted series the first group are inactive against bacteria, whilst the second are active. Whilst it might be postulated that the more positive acridines would have greater opportunity to act as hydrogen acceptors and so become reduced to the feebly ionized dihydro-analogues, it is almost certain that all the above examples have potentials too negative to be inactivated in this way.

Relative Importance of pH in Acridine Action.

It is well known that the acridines are less effective in acidic than in alkaline media (Graham-Smith, 1919; Browning, Gulbransen and Kennaway, 1919; and Eggerth, 1926). Data correlating ionization with activity in the acridine series were not available to these authors, and consequently no entirely adequate explanation was forthcoming on this important reversal of activity. We have been able to extend these observations and to offer an interpretation of the effect of pH on a number of acridines, so selected that changes in pH could be translated in terms of ionization of the drug according to the Law of Mass Action. The theoretical implications of the effect of changing pH on the union of an anion and a kation have been discussed by one of us (Albert, 1944).

Method.—The general procedure of the tests was similar to that described above, except that here a synthetic medium was used throughout. For this purpose a basal medium similar to that described by McIlwain (1941) was adopted, with some minor variations. Appropriate amounts of phosphate buffers were added to give the required pH. All pH measurements were determined with a glass electrode.

TABLE IX.—*The Effect of pH on Ionization and on Antibacterial Activity. Highest Dilutions Completely Preventing Visible Growth in 48 Hours at 37° C.*

Medium : Synthetic medium (see text).

Table.	Number.	Substance.	pH of medium.	Per cent ionization (kation) at 20° C.	Precipitation of drug.	Inhibitory dilutions for—		Relative increase in activity over that at lowest pH against—	
						B. coli.	*Staph. aureus.*	*B. coli.*	*Staph. aureus.*
IV	2	5-Aminoacridine	5·7	100	None	1	1	1	1
			6·8	100	„	4	Not done	8	Not done
			7·5	100	„	6	„	32	„
			8·3	97	„	8	7	128	64
IV	7	2 : 8-Diaminoacridine (proflavine)	5·4	100	„	<1	<1	1	1
			6·7	100	„	2	2	>2	>2
			7·5	100	„	5	6	>32	>64
IV	6	2 : 7-Diaminoacridine	5·7	100	„	1	1	1	1
			6·8	96	„	4	Not done	8	Not done
			7·5	84	„	6	„	32	„
			8·3	44	„	7	6	64	32
V	79	2 : 9-Diaminoacridine	5·4	98	„	2	2	2	2
			7·3	44	Moderate	<1	<1	<1	<1
			8·3	7	Heavy	<1	<1	<1	<1
V	80	3 : 7-Diaminoacridine	5·4	86	None	1	<1	1	1
			6·7	24	„	2	1	2	>1
			7·5	5	Moderate	2	2	2	>2
V	71	3-Aminoacridine	5·4	72	None	1	<1	1	1
			6·7	11	Slight	2	2	2	>2
			7·5	2	Heavy	1	<1	1	1

N.B.—<1 signifies growth in 1 in 5000 dilution, i.e. = 0 of other tables.

The composition of the basal medium was as follows : Ammonium sulphate 0·5 g., ammonium chloride 0·5 g., sodium lactate 2·8 g., ferrous sulphate trace, magnesium sulphate trace, distilled water to 1 litre. Phosphate buffer salts were then added and the medium was autoclaved at 15 lb. pressure for 20 minutes. The final pH was checked as in each case a slight drop in pH invariably followed sterilization.

These media gave good growth with *Bacterium coli*, but with *Staphylococcus aureus* 0·05 per cent of peptone (Parke-Davis & Co.) was added to provide suitable enrichment.

The end-point of bacteriostasis was taken as that dilution completely inhibiting visible growth. In many of the dilutions subnormal growth was noted, when compared to the controls, but these tubes were consistently recorded as positive growth. An unavoidable complication of the experiments was the increased tendency of the less active members to precipitate in the high concentrations necessary to effect bacteriostasis, particularly as the medium was made more alkaline. This rendered a few end-points somewhat uncertain, but did not interfere with the general trend of results.

The findings shown in Table IX disclose an interesting reversal of antibacterial action, the explanation of which can be described in terms of kationic exchange. We see (Table IX) that a 60-fold increase in hydrogen ion concentration (i.e. shift pf pH from 7·5 to 5·7) causes a significant decrease in activity of the strong bases (Nos. 2, 7 and 6), but is without appreciable affect on their degree of ionization. On the other hand, the action of weak bases (Nos. 79, 80 and 71) is unaltered by the same pH shift, but ionization is greatly altered, viz. from poor ionization to almost complete ionization. The results show that under conditions of equal ionization of base (i.e. at pH 5·4) these acridines possess practically equal activity irrespective of their pK_a, whereas under conditions of unequal ionization (i.e. in neutral media) the activity is determined by kationic concentration.

These conclusions may be elaborated further by calculation of the ratio of acridine ions to hydrogen ions necessary to cause bacteriostasis. Table IX (*a*) shows how this ratio is arrived at in several examples, 3- and 5-aminoacridines, and 2 : 7- and 3 : 7-diaminoacridines. These values were obtained with *B. coli*, but those for *Staph. aureus* are entirely comparable.

TABLE IX(*a*).—*Competition Between Hydrogen and Acridine Ions in Synthetic Media.*
(From results with *B. coli* shown in Table IX.)

Substance.	Concentration of acridine ions at inhibitory dilution M × 10^6. pH of medium.				Ratio of acridine ions to hydrogen ions at inhibitory dilution. pH of medium.			
	5·7.	6·8.	7·5.	8·3.	5·7.	6·8.	7·5.	8·3.
5-Aminoacridine	1000	130	32	8·0	510	810	1000	1600
3-Aminoacridine	740*	57	20	Not done	190	285	641	—
2 : 7-Diaminoacridine	950	111	25	6·5	500	690	810	1300
3 : 7-Diaminoacridine	830*	115	18	Not done	210	580	590	—
Average values	880	103	24	7	352	591	760	1450

Average of above = 788.

* pH of medium, 5·4.

It will be seen that from pH 5·7 to 8·3, i.e. over a four-hundredfold change in hydrogen ion concentration, the ratio of acridine ion to hydrogen ion at the inhibitory concentration is relatively constant, varying by only a threefold change. In other words, an increase in the hydrogen ion concentration must be balanced by a like increase in acridine ion concentration for bacteriostasis to occur. This indicates that hydrogen ions are competing with acridine ions for the same position on an enzyme

surface. If we suppose that the hydrogen ion is the normal occupant of this position, which is probably an ionized acidic group, then its displacement by a " foreign " ion will result in inactivation of the enzyme and inhibition of growth. On the evidence here, this inhibition occurs when the acridine ions are about 800 times as numerous as the hydrogen ions.

Any substituent group altering the adsorbability of the acridine kation at the enzyme surface will lower or raise this ratio, depending on whether or not the substituent favours adsorption.

It must be pointed out that the pH near a surface is not necessarily the same as that in the bulk of solution. The charge on the surface (measured as a zeta potential, Hartley and Roe, 1939) attracts a higher concentration of oppositely charged ions to the surface. Although the local change in pH may alter the degree of ionization of the base, there would be no change in the proportion of drug kations to hydrogen ions adsorbed. The factors influencing competition in mineral flotation and their bearing on bacteriostasis have been reviewed elsewhere by one of us (Goldacre, 1944).

Fig. 2 shows pH plotted against the limiting inhibitory concentration of the ions of four typical derivatives. The ionic concentration was calculated from the total inhibitory concentration (i.e. ionized and unionized molecules) and the pK_a value.

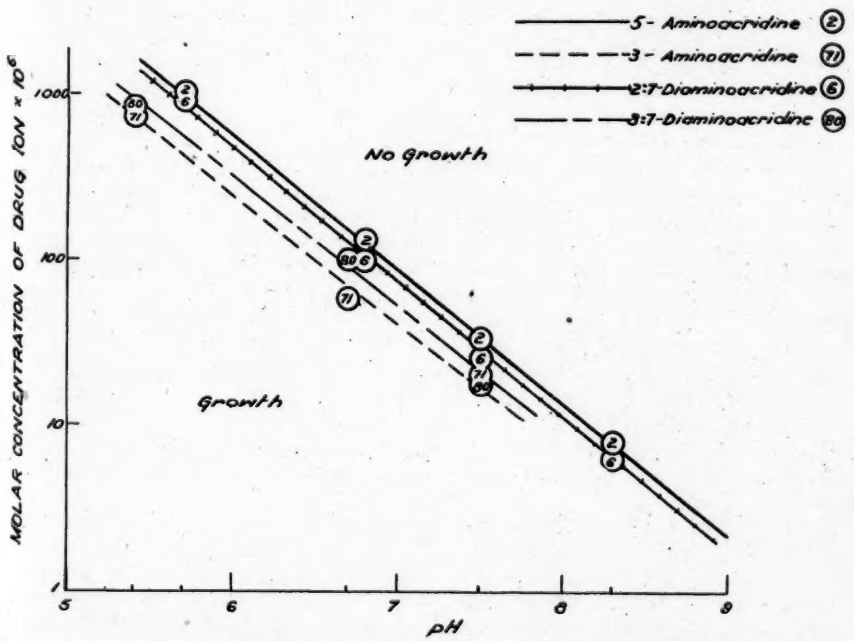

FIG. 2.—Competition between hydrogen ions and acridine ions. Organism = *B. coli*.

While the concentrations of the four compounds in Fig. 2 required to inhibit growth of *B. coli* at pH 7·3 are very different (Tables IV and V), this difference is seen to vanish when the concentration of their *ions* is compared. The slight vertical displacement of the straight lines from one another represents quite small differences in

the bacteriostatic power of the ions under consideration. Above all, the slope of the line is the same for each drug, and it becomes evident that a sixteenfold increase in the hydrogen ion concentration (i.e. a fall of 1·2 pH units) can be compensated for by increasing the drug concentration ten times.

From a line typifying Fig. 2, it is possible to derive an equation from which the limiting bacteriostatic concentration of any simple acridine drug may be derived provided pK_a and pH are known. However, such an equation would be inadequate to deal with the more complicated acridines where dimensional factors or zwitterionic structure, for example, entered.

The Resistance of Gram-positive and Gram-negative Organisms to Aminoacridines.

In all previous experiments (Tables IV, V, VI, VII) it will be noted that the bacteriostatic capacities were determined by using five species of bacteria, three of which were Gram-positive and two Gram-negative. The impression has arisen from this restricted series that the Gram-positive organisms are, on the whole, far more sensitive than are the Gram-negative types. This finding appeared to be fairly adequately explained by the hypothesis of Stearn and Stearn (1924, 1926), who obtained similar results with another kationic series of drugs, the triphenyl-methane dyes. The Stearns' hypothesis is that Gram-positive organisms are more richly endowed with acidic groups than the Gram-negative ones, and hence have a greater affinity for basic dyes. The identity of these groups with the phosphoric acid residues of nucleoprotein is suggested in the recent work of Henry and Stacey (1943).

In order to test the application of this hypothesis in the present series we selected six highly ionized acridines for further examination. To this end the bacteriostatic capacities of the drugs were re-determined for a wider range of Gram-positive and Gram-negative organisms. The results are set out in Table X below.

It will be noted that the average inhibitory dilutions for the Gram-positive types are consistently higher than the dilutions required to inhibit the Gram-negative species, but the difference between the two sets is not striking. Also, amongst the Gram-negatives we can recognize wide variations in susceptibility (*cf. Meningococcus* or *Brucella* with *Ps. pyocyanea*), whereas such variations are not so marked amongst the Gram-positive types. It will be apparent that by a prejudicial selection of certain organisms from Table X the accuracy of Stearns' hypothesis could be either accepted or rejected for this series.

On the other hand, a more convincing analysis of these results may be found, not in the Gram-staining properties, but in the complexity of nutritional requirements of the various species.

The non-exacting species (*Pseudomonas, Proteus, Escherichia*) are the most resistant types, the semi-exacting species (*Eberthella, Vibrio, Staphylococcus*) are less resistant, the exacting species (*Streptococcus, Clostridium, Brucella*) are still less resistant, whilst the very exacting species (*Haemophilus* and *Neisseria*) are the least resistant. In general terms, the exacting nature of an organism's nutritional requirements is gauged by its ability to synthesize gross and fine structural units from simple sources of carbon and nitrogen. As we ascend the scale of increasing complexity in nutrition we find an increasing demand on the part of the organism for special nutrients (special amino acids and growth factors), and, at the same time, an increasing sensitivity to acridine action. Thus it might be suggested that the acridines act adversely on bacteria through interference with the more specialized metabolic activities—a mode of action probably unrelated to Gram staining.

Bernheim (1944) working with *B. coli* found that 5-aminoacridine was a powerful inhibitor of respiration, and hence it is reasonable to suppose that the groups of the organism combining with aminoacridines are intimately related to the respiratory enzymes. A similar conclusion was reached by Martin and Fisher (1944), who found that the bacteriostatic effect of proflavine on staphylococci could be reversed by adenylic acid, diphosphopyridine-nucleotide and several intermediary metabolites, including pyruvic, lactic, acetic, malic, fumaric, succinic and glutamic acids, glucose and phenylalanine.

TABLE X.—*The Sensitivity of Gram-positive and Gram-negative Bacteria towards Certain Aminoacridines. Highest Dilutions Completely Preventing Visible Growth in 48 Hours at 37° C.*

Medium : 10 per cent serum broth, pH 7·2–7·4.

Organism.	Substance.					
	5-Amino-acridine.	1-Methyl-5-amino-acridine.	2-Chloro-7-methoxy-5-amino-acridine.	2 : 8-Di-amino-acridine (pro-flavine).	1 : 9-Di-methyl-2 : 8-di-amino-acridine.	2 : 7-Di-amino-acridine.
Gram-positive species :						
Cl. welchii	7	8	8	6	9	7
Cl. sporogenes	6	7	6	6	8	6
B. subtilis	5	6	5	5	7	5
B. anthracis	6	7	6	5	8	5
Strep. pneumoniae	7	7	7	6	8	6
Strep. pyogenes A	6	7	6	6	8	6
Strep. viridans	6	8	6	6	8	6
Staph. aureus	4	5	5	4	6	4
Lactobacillus	7	7	7	6	8	6
C. diphtheriae	6	7	6	4	7	6
Myco. phlei	6	7	7	6	8	6
Total B.I.	66	76	69	60	85	63
Average B.I.	6	6·9	6·3	5·4	7·7	5·7
Av. inhibitory dilution	1 : 160,000	300,000	200,000	110,000	540,000	140,000
Gram-negative species :						
B. coli (*Escherichia*)	4	4	2	3	4	4
Proteus	4	4	0	2	3	4
Ps. pyocyanea	0	0	0	0	0	0
B. Friedlanderi	5	5	5	3	6	5
B. typhosum (*Eberthella*)	5	6	5	5	5	5
B. dysenteriae Flexner (*Shigella*)	7	7	6	6	7	7
V. cholerae	6	6	6	6	7	6
Pasteurella	6	6	5	3	6	6
Brucella	8	8	7	7	9	7
H. influenzae	7	8	6	7	8	6
N. meningitidis	7	8	7	7	10	7
Total B.I.	59	62	49	49	65	57
Average B.I.	5·3	5·6	4·4	4·4	5·9	5·2
Av. inhibitory dilution	1 : 100,000	130,000	60,000	60,000	150,000	100,000

DISCUSSION.

Physico-chemical aspects.

It may be fairly claimed that a clear understanding is emerging of the connection between constitution and antiseptic action in the acridine series, and that the bridge between constitution and action is to be found in the realm of physical chemistry.

The cumulative evidence derived from 107 acridine compounds as presented in this survey justifies the conclusion that high antibacterial action in this series is, in general, dependent on a basicity sufficient to ensure that most of the substance is present as the kation. This important point is well illustrated in Fig. 3, in which the pK_a of all our compounds is plotted against anti-streptococcal activity, at pH 7·3, the ordinate being the limiting inhibitory concentration of the drug (calculated as base). The points represented by the numbers of the compounds are to be taken as lying at the centre of the circle (or part-circle) around each number. The dotted line is a theoretical curve derived by assuming that, at pH 7·3, (i) a fully ionized acridine would require a minimal inhibitory concentration of 30×10^{-6} molar (as 5-aminoacridine does), and (ii) an incompletely ionized acridine would require a proportionately higher concentration of drug in order to give the same amount of ion as that given by the fully ionized acridine.

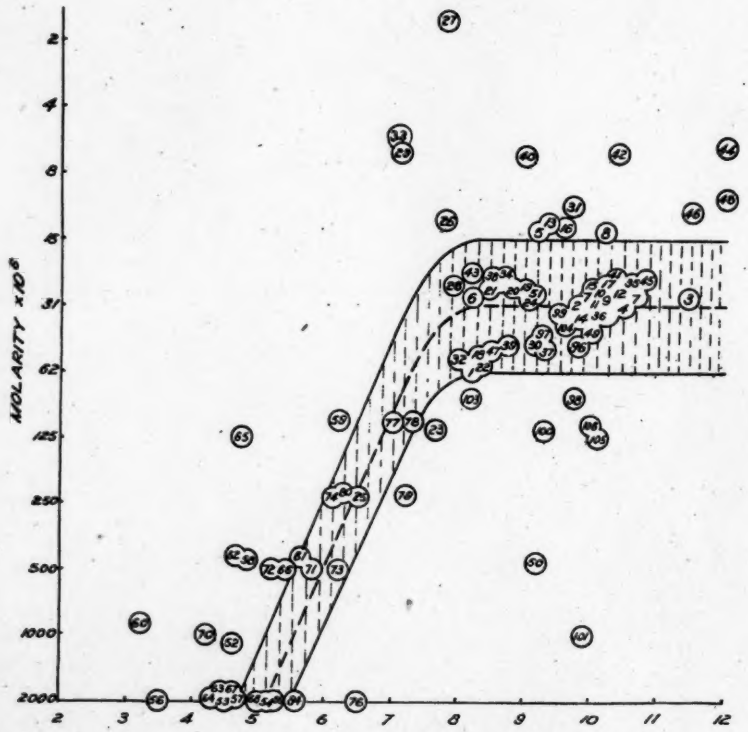

FIG. 3.—The relationship of antibacterial activity to basic strength. Organism: *Streptococcus pyogenes.*

This equi-ionic curve has been expanded to a shaded area in order to accommodate a two-fold variation in end-point, thus embracing the limits of accuracy of the test.

The closeness with which the majority of the points approach, or fall within, the shaded area is an outstanding example of the influence of a physicochemical property in determining antibacterial activity.

Each substance lying outside the shaded area is considered to possess something which hinders or assists the adsorption of the substance on the bacteria, according as it lies below or above the shaded area. Of the *hindered* substances, Nos. 98, 100, 101, 105 and 106 are classified in Table VII and discussed in the text with regard to their steric limitations. No. 50 (Ω-amino-3-methylacridine) probably fails for reasons of chemical instability. Of the *assisted* substances, No. 65 has been discussed as an example of chelation, Nos. 26, 27, 29 and 33 are nitro-derivatives that appear to have a double action, and Nos. 40, 42 and 44 are methyl-derivatives where the adsorption seems to be assisted by the presence of oleophilic methyl groups in appropriate positions in the molecule.

It is worth noting that this curve is a simpler one than that connecting ionization with activity for sulphonamides (Bell and Roblin, 1942), in which activity decreases both above and below 50 per cent. ionization, suggestive of the summation of two opposing factors.

This necessity for adequate ionization is understandable in the light of evidence that there exists a definite competition between hydrogen ions and drug kations for a negatively charged point on the bacterial surface (see Fig. 2). This point is probably the dissociating acidic group of a respiratory enzyme, since both Bernheim (1944) and Martin and Fisher (1944) have related acridine action to respiration. Another type of enzyme seems to be indicated for *B. lactis aerogenes* by Davies, Hinshelwood and Pryce (1944), who find that, in its bacteriostatic range, proflavine acts mainly by prolonging the lag phase of bacterial development through inhibition of the enzyme responsible for the production of the growth-prompting substance normally present in the filtrate from growing bacteria.

Unless each bacterium can grow with only a very few units of its enzyme functioning, it may be claimed that the hydrogen ion, which is the natural occupant of the negatively charged point in question, obviously has a greater affinity for the enzyme than the competing acridine ion has. Thus, for inhibition to occur, acridine ions must be about 800 times more numerous than hydrogen ions (see Fig. 2, and Table IX*a*). This large ratio of drug to natural antagonist is comparable to the figure of 26,000 for sulphanilamide-*p*-aminobenzoic acid antagonism in *Cl. acetobutylicum* (Rubbo and Gillespie, 1942) and of 500 for the pantoyltaurine-pantothenic acid system (McIlwain, 1942), and it stands in contrast to the low figures obtained in some other cases, especially when the drug antagonist reverses inhibitory action by precipitation, e.g. proflavine-nucleic acid with its ratio of 0·1 (unpublished work and McIlwain, 1941).

It would seem that a considerable number of potent acridine antiseptics can at any time be called into being merely by securing what appear to be the two relevant factors : (i) Good ionization at the physiological pH and (ii) a minimum of dimensional hindrance to adsorption. If present-day knowledge of inductive constants be applied to the data herein, it should be possible in most cases to calculate whether a given compound would be sufficiently basic to make its synthesis worth-while. To illustrate this point it could be predicted that the as yet unknown cyano-derivatives of 2-amino-acridine would be unpromising substances, since the cyano-group is more electro-negative than the chloro-group. It will be recalled that the addition of the latter group to 2-aminoacridine resulted in insufficiently ionized substances (see Table V, Nos. 76, 77 and 78).

In a similar manner we may analyse the findings of the various authors who, up to the present, have studied the bacteriostatic properties of 130 acridines. For example, Browning and Calver (1943) reported on a number of derivatives of 5-methylacridine and 5-styrylacridine which, lacking an amino group in the 2-position, would in no case give a pK_a value above 6, and hence it is not surprising that these compounds were inactive. In addition the styryl compounds would be handicapped by the dimensional factor. These authors examined the corresponding quaternary salts (10-methochlorides, etc.), which would be well ionized and moreover stabilized by inert substitution in the 5-position. As a result it was found that the quaternary salts derived from 5-methylacridines had considerable activity but those derivatives from the 5-styrylacridines were inferior, probably because the dimensional factor still operated. A close inspection of the other published results reveals in every case findings which are concordant with the hypothesis elaborated here.

Recently one of us (Albert, 1941) proposed the name " Kationic Antiseptics " as a collective term for salts having a kation of relatively high molecular weight which owed their antibacterial action to an ability to form feebly ionized complexes with acidic groups in the organisms. The question now arises : Are the aminoacridines only a special and favourably constructed sub-class of the known kationic antiseptics, or have the best examples a " specific acridine action " in addition ? We do not think that this question can be definitely answered at this juncture, but there is this to be said in favour of a specific action : No other group of common kationic substances shows the same intensity of action against a wide range of organisms in the presence of serum protein as do the 2- and 5-aminoacridines and their derivatives. This high potency is probably due to those structural features in the acridine molecule which make for ease in adsorption on bacterial enzymes and for difficulty in adsorption on serum proteins (Table VIII). Thus the whole of the drug remains available for antibacterial action. Ease of adsorption on the bacterial enzymes evidently requires that a molecule should be flat or at least flexible, and it is here that 5-amino-tetrahydroacridine (Fig. 1) fails ; but it is apparently equally necessary that a minimum molecular size be reached, and this explains why 4-aminoquinoline (Fig. 1) is inactive (Albert, 1944). This requirement of size is to provide a sufficiently great area for adsorption ; it depends on the fact that increase in the number of atoms in a flat molecule increases its Van der Waals attraction, whilst the kinetic energy of translation, which is the force tending to remove the molecule, is independent of molecular size. The fact that one can place substituents in the acridine nucleus in special positions, where they will increase the activity of a fully ionized substance (*cf.* the effect of inserting two methyl groups into the 1- and 9-, but not the 3- and 7-positions of proflavine) suggests the presence of receptor sites on the organism peculiarly suited for adsorbing the acridine nucleus. The same conclusion may be drawn from the fact that substituents increasing bulk beyond certain dimensions have an inactivating influence (Table VII).

On the other hand, 5-amino-2 : 3-benzacridine (Fig. 1) is not too large to be fully active, and even the angular benzoquinolines (e.g. 4-aminobenzo-h-quinoline, Fig. 1), which we are now investigating, show an activity only slightly less than that of the corresponding aminoacridines if fully ionized examples are compared. All these facts suggest that the aminoacridines are specific in their action only in the sense that the relevant bacterial enzymes are vulnerable, in the presence of serum, to kations of the general dimensions and physical properties of acridine itself. In contradistinction to the acridines, the aliphatic amines (e.g. " Cetavlon ") and the triphenylmethanes (e.g. crystal violet), which together form the bulk of the best-known of the non-acridine kationic antibacterials, are adsorbed on serum protein at the expense of bacteriostasis (unpublished work).

Recently Valko and DuBois (1944) concluded that the action of acriflavine could be reversed by sodium dodecyl sulphate, which also reversed the action of surface active kationic substances, such as " Zephiran." This was brought forward as evidence that the action of all kations can be satisfactorily described in terms of kationic exchange by the bacteria, without invoking a specific action on the part of the drug. We are in general agreement with these conclusions, but it appears to us, on reproducing their experiments, that the reversal of acriflavine or " Zephiran " was due partly to precipitation of the antiseptic by the reversing agent. A similar type of reversing action by precipitation was reported on by McIlwain (1941) using nucleic acid and acriflavine.

On the other hand, McIntosh and Selbie (1943) showed that staphylococci made resistant to proflavine are also resistant to propamidine, but are not resistant to 2 : 7-diaminoacridine or 5-aminoacridine. Further, staphylococci could not be made resistant to 5-aminoacridine and only with difficulty to 2 : 7-diaminoacridine. On these facts McIntosh and Selbie suggested different modes of action for different acridines, influenced mainly by the presence of substituent groups. We find, however, that 5-aminoacridine does not potentiate the action of proflavine or 2-aminoacridine, which might be expected if they were acting differently. The differences in acquiring resistance may only be a measure of the facility of the trained organism to destroy (by oxidation or deamination) the drug to which it is resistant.

5-Aminoacridine is an extraordinarily stable substance and staphylococci apparently cannot be trained to destroy it, so that increased resistance is impossible. We agree with these authors that substituents are important in so far as they affect ionization or facilitate destruction of the kation. Yet another hypothesis concerning resistance is furnished by Davies, Hinshelwood and Pryce (1944), who believe that the acquired resistance of *B. lactis aerogenes* to proflavine depends on the production of an excess of the enzyme blocked by this acridine.

The question may be raised as to the role of ionic resonance in the antibacterial action of aminoacridines. This is, of course, but one aspect of the current discussion on the general importance of resonance in biochemical processes, and we can only refer to the views expressed by Kalckar (1941), Szent-Gyorgi (1941), Huggins (1943) and Kumler and Daniels (1943). The simplest explanation is that ionic resonance may increase specific adsorption on the essential bacterial enzyme, firstly by flattening the entire molecule and secondly by facilitating its union, by primary and secondary valencies, with resonant structures on the enzyme surface. As we have succeeded in preparing highly ionized derivatives lacking in ionic resonance but possessing marked antibacterial action, our tentative conclusion is that resonance is not essential in contributing to action in the acridine series (Table IV, Nos. 49 and 51). We cannot agree with Kumler and Daniels (1943) that a doubly-bound, positively charged, primary amino group ($=N^+H_2$) plays the same role in determining biological activity of acridines that it may well do in the case of the sulphonamides.

The dimensional factor is not so readily defined, but as a working hypothesis it may be assumed that the addition of a side-chain of more than four atoms (excluding hydrogen) or the hydrogenation of one or more of the benzene rings can be prejudicial to activity.

Finally, it has been shown that the most active derivatives are amongst those least attracted to oil, which is consistent with our view that aminoacridines act in the watery phase in contact with the bacterial enzymes. This might suggest that the external surface of the bacterial membrane is the most probable site of action of these substances, for there is an extensive literature on the relative difficulty of ions (compared to neutral molecules) in penetrating membranes (*cf.* Davson and Danielli, 1943 ; Kumler and Daniels, 1943). The requirements of reduction-potentials cannot yet be

specified numerically, but it is almost certain that these requirements cannot seriously be transgressed in the acridine series if the previously mentioned rules are observed.

Clinical aspects.

Whilst our main interest in this work has been the influence of chemical constitution in antibacterial activity, we have been keenly aware of the clinical potentialities of certain members of the present series. The well authenticated clinical findings obtained since 1917, and particularly during the present war, can leave no doubt as to the efficacy of aminoacridines as surface antiseptics. Three entirely new acridines have been described here which present such striking advances that they are worthy of separate comment. The drugs in question are : 1-methyl-5-aminoacridine (Tables IV and X, No. 8) ; 1 : 9-dimethyl-proflavine (Tables IV and X, No. 40) ; 2-nitro-5-aminoacridine (Table IV, No. 27).

1-*Methyl-5-aminoacridine.*—This substance is suggested as possessing certain advantages over 5-aminoacridine, which was previously recommended by us as a non-staining acridine. 1-Methyl-5-aminoacridine is significantly more active than the parent molecule against Gram-positive and Gram-negative bacteria (Table X), it is less toxic (L.D. 50 = 0·13 g./kg. mouse, subcutaneously), non-staining, and has not the very bitter taste characteristic of 5-aminoacridine. The indications for its use would be the same as those for 5-aminoacridine.

1 : 9-*Dimethyl-proflavine.*—This drug, with the possible exception of 2-nitro-5-aminoacridine, is the most active aminoacridine so far discovered. The introduction of two methyl groups has enhanced antibacterial action over that of proflavine approximately fivefold for Gram-positive organisms, and twofold for Gram-negative types (Table X). This significant increase in activity is not accompanied by a corresponding increase in toxicity (L.D. 50 = 0·09 g./kg. mouse, subcutaneously). In view of these comparisons it can be expected that 1 : 9-dimethyl-proflavine may prove even more useful than the other coloured acridines (" flavines ") hitherto available.

2-*Nitro-5-aminoacridine.*—Apart from its general activity the outstanding feature of this substance is its marked antistreptococcal action, which is manifested in a dilution of 1 in 2,500,000. This property suggests a sphere of usefulness in treatment of haemolytic streptococcal infections of the throat, skin, wounds and prophylactically in obstetrics. The drug is relatively non-toxic and poorly staining.

The experiments describing the reversal of acridine action with increasing hydrogen ion concentration have not only an academic significance, but can be well applied clinically. For the development of fullest activity within physiological limits the aminoacridines should be used at pH 7·3, and if applied to a suppurating wound, which will sometimes have a slightly acid pH (e.g. 6·0), it becomes desirable to neutralize and buffer the wound during acridine therapy. Irrigation of the wound with saline sodium bicarbonate solution would therefore seem more desirable, as a preliminary treatment, than the usual saline irrigation now in common use. Furthermore, neutralized proflavine solutions should always be used in place of acidic proflavine sulphate (Albert and Gledhill, 1943). Where powder therapy is indicated mixtures such as proflavine sulphate 1 per cent in sulphathiazole could be improved by the addition of 1 per cent of heavy magnesium oxide.

SUMMARY.

1. One hundred and seven acridine compounds, 77 of which are new, have been synthesized for this survey, and have been graded according to their bacteriostatic capacities. Their antibacterial activity has been traced back to certain underlying physical and chemical properties.

2. It is shown that a basicity sufficient to induce at least 75 per cent. ionization at pH 7·3 at 20° C. (or 60 per cent. at 37° C.) is essential for effective bacteriostasis in this series. This means that an acridine derivative with a pK_a less than 7·8 is almost certain to be ineffective against bacteria (see Tables IV and V, and Fig. 3).

3. Furthermore, it is shown that the activity of acridine resides mainly in the kations, and that anions, zwitterions and undissociated molecules have little activity (Tables V and VI).

4. Evidence is brought forward that acridine kations injure bacteria by competing with hydrogen ions (for a vital enzyme position), much as sulphonamides injure bacteria by competing with *p*-aminobenzoic acid. In the present case this competition presumably concerns vital acidic groups on the enzymes, which ionize well only in neutral or alkaline media, and it is shown how variations in the pH of the environment will weight this competition for or against the drug (Tables IX and IX*a*).

5. The importance of maintaining size and shape of acridine derivatives within certain dimensional limits is discussed in respect to the influence of these factors on bacteriostatic capacity (see Table VII).

6. Substituents in the acridine nucleus exert their effect principally through controlling ionization and less importantly through facilitating specific adsorption at enzyme sites. Specific adsorption, contrasted against generalized adsorption on protein, is shown to be a feature of this series (see Table VIII).

7. The oil/water partition coefficients of a selected number of the most active members of this series are not far removed from unity, and it is deduced from this that antibacterial action takes place in the aqueous phase (see Table VIII).

8. The significance of ionic resonance and of the very negative reduction potentials of aminoacridines is outlined.

9. The bearing of the present investigations on the clinical use of acridines is discussed and three new drugs are recommended for clinical trial, namely, 1 : 9-dimethyl-proflavine, 1-methyl-5-aminoacridine, 2-nitro-5-aminoacridine.

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REFERENCES.

Albert, A.—(1941) *Lancet*, ii, 633.—(1944) *Aust. J. Sci.*, **6**, 137.
Idem, Francis, A., Garrod, L. P., and Linnell, W. H.—(1938) *Brit. J. exp. Path.*, **19**, 41.
Idem and Gledhill, W.—(1943) *Lancet*, ii, 238.
Idem and Goldacre, R.—(1943) *J. chem. Soc.*, 454.
Idem, Goldacre, R., and Heymann, E.—(1943) *Ibid.*, 651.
Idem and Ritchie, B.—(1943) *Ibid.*, 458.
Idem, Rubbo, S. D., and Goldacre, R.—(1941) *Nature*, **147**, 332.
Alles, G.—(1944) *Univ. Calif. Publ. Pharm.*, **2**, 161.
Bell, P., and Roblin, R.—(1942) *J. Amer. chem. Soc.*, **64**, 2905.
Bernheim, F.—(1944) Personal communication.
Berry, H.—(1941) *Quart. J. Pharm.*, **14**, 363.
Branch, G., and Calvin, M.—(1941) ' The Theory of Organic Chemistry.' New York, pp. 195, 282.
Breyer, B., Buchanan, G., and Duewell, H.—(1944) *J. chem. Soc.*, 360.
Browning, C., and Calver, K.—(1943) *Ibid.*, 345.
Browning C., Gulbransen, R., and Kennaway, E.—(1919) *J. Path. Bact.*, **23**, 106.

DAVIES, D., HINSHELWOOD, C., AND PRYCE, J.—(1944) *Trans. Faraday Soc.*, **40**, 397–420.
DAVSON, H., AND DANIELLI, J.—(1943) ' Permeability of Natural Membranes.' Cambridge, Chapter 14.
EGGERTH, A. H.—(1926) *J. infect. Dis.*, **38**, 440.
GARROD, L. P.—(1935) *Ibid.*, **57**, 247.
GOETCHIUS, G., AND LAWRENCE, C.—(1944) *J. Lab. clin. Med.*, **29**, 1177.
GOLDACRE, R.—(1944) *Aust. J. Sci.*, **6**, 112.
GRAHAM-SMITH, G.—(1919) *J. Hyg., Camb.*, **18**, 1.
GREEN, H., AND BIELSCHOWSKY, F.—(1942) *Brit. J. exp. Path.*, **23**, 13.
HALL, N., AND SPRINKLE, M.—(1932) *J. Amer. chem. Soc.*, **54**, 3469.
HARTLEY, G., AND ROE, J.—(1939) Faraday Society Discussion, " The Electrical Double Layer," p. 101.—(1940) *Trans. Faraday Soc.*, **36**, 105.
HENRY, H., AND STACEY, M.—(1943) *Nature*, **151**, 671.
HUGGINS, M.—(1943) *Chem. Rev.*, **32**, 195.
KALCKAR, H.—(1941) *Ibid.*, **28**, 71.
KUMLER, T., AND DANIELS, W.—(1943) *J. Amer. chem. Soc.*, **65**, 2190.
MCILWAIN, H.—(1941) *Biochem. J.*, **35**, 1311.—(1942) *Brit. J. exp. Path.*, **23**, 95.
MCINTOSH, J., AND SELBIE, F. R.—(1943) *Ibid.*, **24**, 246.
MARTIN, G., AND FISHER, C. V.—(1944) *J. Lab. clin. Med.*, **29**, 383.
MIZUTANI, M.—(1925) *Z. phys. Chem.*, **118**, 327.
RUBBO, S. D., ALBERT, A., AND MAXWELL, M.—(1942) *Brit. J. exp. Path.*, **23**, 69.
RUBBO, S. D., AND GILLESPIE, J.—(1942) *Lancet*, i, 36.
SHARP, W., SUTHERLAND, M., AND WILSON, F.—(1943) *J. chem. Soc.*, 344.
SIDGWICK, N.—(1937) ' The Organic Chemistry of Nitrogen.' Oxford.
STEARN, A., AND STEARN, E.—(1924) *J. Bact.*, **9**, 491.—(1926) *Ibid.*, **11**, 345.
SZENT-GYORGI, A.—(1941) *Nature*, **148**, 157.
VALKO, E., AND DUBOIS, A.—(1944) *J. Bact.*, **47**, 15.
ZENTMEYER, G.—(1944) *Science*, **100**, 294.

AGE AND DOSAGE IN THE INDUCTION OF BREAST CANCER IN MICE BY THE MOUSE MAMMARY TUMOUR AGENT.

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BITTNER (1940*a*) showed that 4–6 weeks old high cancer strain mice which had been foster nursed by low cancer strain mothers developed a high incidence of breast cancer when they were given milk of high cancer strain females which contains the milk influence. Susceptible mice which had not obtained the milk influence while nursing, if permitted to have several litters before they received the milk of high cancer strain females, developed only very few breast tumours (Bittner, 1942*a*, *b*). Bittner concluded, therefore, that the milk influence has to be present from the time the mammary glands start to develop in order to prepare them for the cancerous change and induce a high incidence of breast cancer (Bittner, 1942*b*). He, therefore, proposed for the milk influence the name " preparatory mammary tumour influence or inciter." It is proposed to refer in this paper to the milk influence as the mammary tumour agent.

The findings of Bittner (1940*b*) and Andervont (1939, 1941) established a relationship between the amount of milk received from high cancer strain females and the incidence and average tumour age of breast cancer in mice.

Following these observations experiments were undertaken to ascertain whether it is possible by administering sufficient amounts of material from high cancer strain mice containing the mammary tumour agent to induce breast cancer in susceptible mice, which had not obtained the agent in milk while suckling, when their mammary glands were already fully developed in the absence of the agent.

METHODS.

R III and Strong A breast tumour tissue, dried over phosphorus pentoxide in a vacuum chamber and stored for a period of 2–4 weeks in the ice-chest, was used as the source of the agent. The dried breast tumour tissue was resuspended, immediately before use, in distilled water in a dilution of 1 : 4 and injected subcutaneously. Each mouse was given at each injection 0·5 c.c. of the suspension containing 0·125 g. of breast tumour tissue. It had been found previously that this dilution represents the maximum amount of tumour tissue that can be injected subcutaneously without causing ulcerations, and that it is absorbed entirely within a week. The injections, therefore, were given at weekly intervals.

C 57 female × Strong A male hybrid mice were employed in the present experiments as test mice as, in our experience, these hybrid mice are susceptible to the induction of breast cancer. No spontaneous breast cancer has occurred as yet in C 57 × Strong A females in our laboratories. The incidence of breast cancer in our Strong A high cancer strain females, as reported in a previous paper (Dmochowski, 1944), is about 96 per cent at an average age of 12·6 months, while no breast cancer has as yet been observed in the C 57 black females maintained in our laboratories.

These hybrid mice were employed in the present experiments because they had been suckled by C 57 low cancer strain mothers and presumably had received no mammary tumour agent in the milk of their mothers, but had inherited the genetic factor for susceptibility from their Strong A high cancer strain fathers. They have the further advantage of being vigorous animals, and live for periods considerably longer than 20 months.

The C 57 × Strong A hybrid females of each litter were marked individually and divided as equally as possible into experimental and control mice. They were divided into two groups according to age (Fig. 1). The first group comprised females 4–6

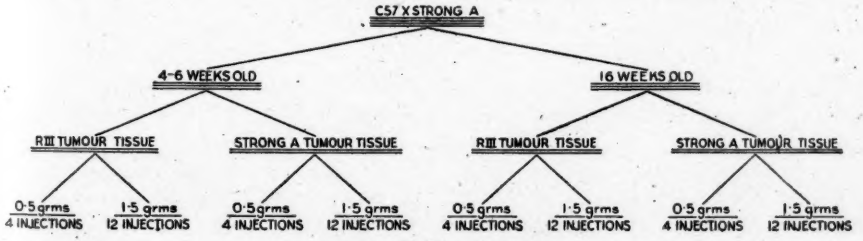

FIG. 1.—Division of mice.

weeks of age, the second females 16 weeks of age which had given birth to one or two litters. Each of the two groups of C 57 × Strong A mice was divided into two groups according to the origin of the mammary tumour agent administered. Mice of the first group received R III breast tumour tissue; mice of the second group received Strong A breast tumour tissue.

Each of the two groups of C 57 × Strong A mice, 4–6 weeks and 16 weeks of age, subdivided into two groups according to the origin of the mammary tumour agent

given, were in turn separated into two groups ; each mouse of the first group received four injections comprising altogether 0·5 g. of breast tumour tissue ; each mouse of the second group received twelve injections comprising together 1·5 g. of breast tumour tissue (R III or Strong A tumour tissue).

The administration of breast tumour tissue containing the mammary tumour agent to all groups of mice was started in January, 1943. All females were forcibly bred (at least three litters in rapid succession) and kept under similar conditions on a diet of " rat-cake " cubes with an unlimited supply of tap-water.

RESULTS.

The results of the experiment are summarized in Tables I and II.

The appearance of the tumours in successive months after the administration of the mammary tumour agent is shown in Table I (R III dried breast tumour tissue) and in Table II (Strong A dried breast tumour tissue).

TABLE I.—*Appearance of Breast Tumours in C 57 × Strong A Female Mice after Administration of R III Dried Tumour Tissue.*

Number of mice.	Age of mice in weeks.	Number of injections.	Number of breast tumours appearing monthly.											Tumours.		Average tumour age in months.
			11.	12.	13.	14.	15.	16.	17.	19.	20.	21.	26.	Number.	%.	
7	16	12	..	..	1	1	..	..	1	..	1	..	1	5	83%*	18
10	4–6	12	..	1	1	..	4	..	1	..	1	..	..	8	80%	15·2
12	16	4	..	..	..	..	..	..	..	..	..	..	..	0	0%	..
15	4–6	4	1	4	1	1	3	1	..	1	1	1	..	14	93%	14·7

* One mouse in the first group died before tumour age.

As can be seen from Table I the administration of 1·5 g. of R III breast tumour tissue induced a high incidence of breast cancer in 4 months old C 57 × A susceptible females which had previously littered. No tumours occurred in their litter-mate controls of the same age when the dose of R III breast tumour tissue containing the mammary tumour agent was smaller. There was little difference in the tumour incidence and the average tumour age in mice 4–6 weeks old, whether given 0·5 g. or 1·5 g. of R III breast tumour tissue. No tumours were observed in litter-mate control mice although they lived for 29 months.

TABLE II.—*Appearance of Breast Tumours in C 57 × Strong A Female Mice after Administration of Strong A Dried Tumour Tissue.*

Number of mice.	Age of mice in weeks.	Number of injections.	Number of breast tumours appearing monthly.											Tumours.		Average tumour age in months.
			12.	13.	15.	16.	17.	18.	19.	20.	21.	22.	28.	Number.	%.	
10	16	12	..	..	..	..	..	2	..	1	1	2	..	6	60%	20
11	4–6	12	1	1	4	..	1	..	..	1	..	..	..	8	72%	16·7
10	16	4	..	..	..	..	..	..	..	..	..	..	1	1	10%	28
8	4–6	4	..	..	2	1	..	..	1	..	1	..	..	5	62%	17

As can be seen from Table II, similar results were obtained after the administration of 1·5 g. of Strong A breast tumour tissue which induced a high incidence of breast cancer in 4 months old females which had previously given birth to one or two litters. One tumour developed in their litter-mate controls after the administration of a smaller dose of Strong A breast tumour tissue containing the mammary tumour agent. The results obtained after the administration of Strong A breast tumour tissue to 4–6 weeks old females were similar to the results obtained with R III breat tumour tissue.

The tumour incidence and the average tumour age were similar whether the 4–6 weeks old mice were given 12 or 4 injections of Strong A breast tumour tissue. No tumours developed in the litter-mate control mice.

DISCUSSION.

Andervont (1940) pointed out that a definite amount ("threshold dose") of the milk of high cancer strain females is essential for the induction of breast cancer in mice. He showed (Andervont, 1939, 1940) that the incidence of breast cancer in high cancer strain females foster nursed by low cancer strain mothers depends on the time when the newborn mice are taken away from their high cancer strain mothers and given to low cancer strain foster mothers. The longer the newborn mice remain with their own mothers the earlier the tumours appear and the higher is the breast cancer incidence. The incidence and also the time of appearance of breast cancer depend, therefore, on the amount of milk obtained from high cancer strain females.

Bittner (1942*a*, *b*) stated that the structure of the mammary glands of mice which have received the milk of high cancer strain females differs from that of mice suckled by low cancer strain mothers. Van Gulik and Korteweg (1940) first observed that the characteristic structure of mammary glands of high cancer strain and low cancer strain mice may be altered as the result of foster nursing. These observations have been confirmed by Shimkin, Grady and Andervont (1941) on other high and low cancer strains of mice. According to Bittner (1942*a*) the milk agent induces a high incidence of breast cancer when supplied to susceptible mice before their mammary glands are fully developed, or to use Bittner's expression, "before the structure of the mammary glands is established." Afterwards, when the mammary glands are fully developed in the absence of milk agent as in mice which have littered, the milk agent induces only very few tumours. "Thus," according to Bittner, "the oestrogenic hormones and the active milk influence would play similar roles in the etiology of mammary cancer in mice."

Andervont, Shimkin and Bryan (1942) have shown that the older the mice, the less susceptible they are to the action of the mammary tumour agent present in the milk of high cancer strain females. Mice up to 23 days old developed 69 per cent of breast tumours when fed or injected with milk of high cancer strain females; mice 30–57 days old although fed or injected with double the amount of milk developed only 14 per cent of breast tumours, and mice 3–4 months old fed with four times the amount of milk developed only 8 per cent of tumours. They suggested that the low incidence of mammary cancer in the older mice results from the destruction of the agent in the gastro-intestinal tract of older mice.

The present experiments have demonstrated that the age of the mice determines their susceptibility to the action of the mammary tumour agent present in the breast tumour tissue of mice. Since 4–6 weeks old mice injected with either 0·5 g. or 1·5 g. of R III, or Strong A tumour tissue containing the mammary tumour agent, gave a similar tumour incidence at the same average tumour age, it can be concluded that 0·5 g. of breast tumour tissue is more than the minimum effective dose.

There exists, however, a difference between the action of R III and Strong A breast tumour tissue if given in four injections of tumour tissue to 4–6 weeks old C 57 × Strong A mice which were litter mates. The tumour incidence after the administration of R III tumour tissue is higher (93 per cent) and the average tumour age is much lower (14·7 months) than after the administration of the same amount of Strong A tumour tissue (62 per cent at the average of 17 months). There is no significant

difference in the tumour incidence after 12 injections of R III or Strong A tumour tissue, although the time of tumour appearance is earlier after the administration of R III tumour tissue.

While mice 16 weeks of age did not develop breast cancer if given 0·5 g. of R III tumour tissue, one breast tumour developed in their litter mates after the administration of 0·5 g. of Strong A tumour tissue. Mice 16 weeks of age when injected with 1·5 g. of R III tumour tissue gave a high incidence of breast cancer although at a later average age than the young mice. In these mice also the tumour incidence was higher (83 per cent) and the average tumour age lower (18 months) after the administration of R III tumour tissue than after the administration of Strong A tumour tissue (60 per cent at the average of 20 months).

These results show that it is possible to induce a high incidence of breast cancer in susceptible mice which have mammary glands already fully developed in the absence of the mammary tumour agent present in milk of high cancer strain females. It is not necessary, therefore, for the agent to take part in the development of mammary glands of susceptible mice to induce a high incidence of breast cancer. The high incidence of breast cancer induced in mice which have littered with large quantities of the breast tumour tissue containing the mammary tumour agent is similar to the incidence of breast cancer induced by the agent in smaller amounts of the breast tumour tissue in young mice. A similar incidence has been reported by Andervont, Shimkin and Bryan (1942) in 10 days old mice after one administration of milk of high cancer strain females.

The results of the present experiments demonstrate that the tumour-inducing activity of R III breast tumour tissue is greater than that of Strong A breast tumour tissue, although the R III tumour tissue was administered to C 57 × Strong A mice which have a different genetic constitution from R III strain mice. At present it is not known whether the difference in the tumour-inducing activity of R III and Strong A dried tumour tissues results from differences in the potency of the mammary tumour agent they contain, or whether it depends on varying amounts of the same agent present in tumours of these two strains. The results are thus explicable on either qualitative or quantitative considerations.

These results also confirm previous findings (Dmochowski, 1944) that breast cancer can be induced in susceptible mice which are of an entirely different genetic constitution from the strain from which the mammary tumour agent was obtained. In this case the mammary tumour agent present in resuspended S × R III breast tumour tissue induced breast cancer in C 57 × Strong A hybrids.

The findings of the present investigations are pertinent in assessing the relative significance of the role of oestrogenic hormones and of the mammary tumour agent in the induction of breast cancer in susceptible mice. Few or no tumours have been induced by the administration of oestrogens in susceptible mice after they have been nursed by low cancer strain females—that is, when their mammary glands have already developed in the absence of the mammary tumour agent (Bittner, 1941 ; Shimkin and Andervont, 1941 ; Dmochowski and Gye, 1944). A high incidence of breast cancer, however, can be induced by large doses of breast tumour tissue containing the mammary tumour agent in susceptible mice nursed by low cancer strain mothers, although their mammary glands are already fully developed in the absence of the agent as shown in the present experiments.

Therein may be the difference between the action of oestrogenic hormones and the mammary tumour agent in the development of breast cancer in mice. While the oestrogens can only induce breast cancer in susceptible mice if the development of mammary glands has taken place in the presence of the mammary tumour agent, the

latter can exert its action in susceptible mice even after the mammary glands have developed in its absence.

SUMMARY.

A high incidence of breast cancer has been induced by administering strong A and R III dried breast tumour tissue to 4 months old hybrid female mice (C 57 × Strong A), in which the mammary glands had already completed their development in the absence of the mammary tumour agent. Older mice require larger quantities of tumour tissue containing the mammary tumour agent than do young mice in order to induce the same tumour incidence.

Differences between the tumour-inducing activity of R III and Strong A breast tumour tissue have been demonstrated. R III tumour tissue induces a higher tumour incidence at a lower age than does Strong A tumour tissue.

The relative role of oestrogenic hormones and mammary tumour agent in the development of breast cancer in mice is discussed.

REFERENCES.

ANDERVONT, H. B.—(1939) *Publ. Hlth. Rep.*, **54**, 1597.—(1940) *J. nat. Cancer Inst.*, **1**, 147.—(1941) *Ibid.*, **2**, 13.

Idem, SHIMKIN, M. B., AND BRYAN, W. R.—(1942) *Ibid.*, **3**, 309.

BITTNER, J. J.—(1940*a*) *Ibid.*, **1**, 155.—(1940*b*) *Proc. Soc. exp. Biol., N.Y.*, **45**, 805.—(1941) *Cancer Res.*, **1**, 290.—(1942*a*) *Ibid.*, **2**, 710.—(1942*b*) *Ibid.*, **2**, 755.

DMOCHOWSKI, L.—(1944) *Brit. J. exp. Path.*, **25**, 138.

Idem AND GYE, W. E.—(1944) *Ibid.*, **25**, 115.

SHIMKIN, M. B., AND ANDERVONT, H. B.—(1941) *J. nat. Cancer Inst.*, **1**, 599.

SHIMKIN, M. B., GRADY, H. G., AND ANDERVONT, H. B.—(1941) *Ibid.*, **2**, 65.

VAN GULIK, P. J., AND KORTEWEG, R.—(1940) *Proc. Soc. Nederl. Akad. v. Wetenschappen.*, **43**, 891. (Quoted by Bittner, J. J., in *Cancer Res.* (1942), **2**, 710.)

THE PRODUCTION, PURIFICATION AND TITRATION OF THE NEUROTOXIN OF *SHIGELLA DYSENTERIAE*.

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WITHIN a few years of its discovery (Shiga, 1898) the toxic character of *Shigella dysenteriae* had been recognized by a number of workers, and from the early days there was controversy as to whether one or two toxic substances were responsible for the symptoms of dysentery observed. The subject is well reviewed by Weil (1943), and it is only necessary to repeat a few of the findings which he has collated. Boivin and Mesrobeanu (1937*a*, *b*, *c*, *d*, *e*, *f*) proved beyond reasonable doubt that two toxins are produced by *S. dysenteriae*. One is a protein obtained by precipitation with trichloroacetic acid at pH 3·5, and the other a complex of polysaccharide + phospholipin + protein, obtained by dialysis and precipitation with ethanol, from the solution remaining after removal of the toxic protein.

The protein, formed in about equal amounts by both "Rough" and "Smooth" strains, was shown to be responsible for the nervous and paralytic symptoms developed by the rabbit after injection with Shiga's bacillus and is now described as the neurotoxin. The polysaccharide complex, identical with the O somatic antigen and present

only in " Smooth " organisms, provoked the diarrhoea and other intestinal symptoms and has been named the enterotoxin. Boivin and Mesrobeanu showed that the enterotoxin and the neurotoxin were present in " Smooth " organisms, but not in the filtrate from very young cultures. In old broth cultures the neurotoxin is found in the filtrate, together with a small amount of enterotoxin. They showed that the neurotoxin gave no reaction with antibacterial sera prepared by use of either " Rough " or " Smooth " organisms, but flocculated with, and was neutralized by, Shiga antitoxic sera. The neurotoxin, but not the enterotoxin, can be converted into toxoid by the action of formaldehyde.

Sporadic attempts have been made since Shiga's early experiments (1903) to immunize man against bacillary dysentery. Recently polyvalent vaccine (Forsyth, 1942), combined polyvalent vaccine and toxoid (Clauberg and Sartorius, 1942 ; Kestermann and Vogt, 1941), and endotoxin-toxin-antitoxin (ETA) (Prigge, 1937, 1940), have been used on a small scale with encouraging results. Farrell and Ferguson (1943), and Farrell, Fraser and Ferguson (1944) have reported experiments using Shiga toxoid in which reasonable antibody response in man was observed.

The work described here was undertaken partly to increase our knowledge of the neurotoxin, and partly to improve the preparation of an antigen suitable for the immunization of horses to produce antitoxin for therapeutic use and for human prophylaxis.

Titration of the Neurotoxin.

The methods used to follow the production and purification of the toxin were to determine the combining power with antitoxin by *in vivo* and *in vitro* titrations, with a comparison of the results yielded by the two methods.

In vivo titration.—For the *in vivo* titrations " Laboratory standard " sera were used which had been evaluated by comparison with the 200 unit/ml. British Standard anti-neurotoxin. The experiments were arranged so that mice received either one or five units of antitoxin and graded doses of toxin. As an illustration the procedure at the " one unit level " will serve.

In a series of tubes are placed (*a*) increasing volumes, from 0·30 to 1·50 ml., of the toxin to be titrated, (*b*) sufficient 0·9 per cent sodium chloride in borate buffer solution at pH 8·0 to make the volume up to 1·5 ml. ; (*c*) 1 ml. of serum dilution (in borate buffer saline) containing five units of antitoxin per millilitre. Thus 2·5 ml. of the final mixture contains 5 units of antitoxin, i.e. 1 unit/0·5 ml. After mixing by inversion, the mixtures are allowed to stand for 1 hour. A small number of mice (usually two or three) is injected intravenously with 0·5 ml. amounts of each mixture, i.e. with varying amounts of toxin plus one unit of antitoxin. The symptoms and deaths among the mice are noted over a period of 5 days. The test is usually carried out once with 50 per cent differences between toxin doses and twice with 20 per cent differences. The dose for which the fourth day is the average day of death is taken as the end-point. The amount of toxin in that dose is regarded as the $L +$ *dose at the "one unit level."*

In order to obtain consistent results it is necessary to use healthy mice weighing between 16 and 20 g. A slight excess of toxin usually causes death on the third or fourth day after injection. Deaths occurring before the fourth day may be caused by larger doses of toxin or by the use of unhealthy mice which succumb to the shock of injection. Dysentery toxin seems to cause this " non-specific shock " death more readily than most other toxins. This difficulty was noted by Blake and Okell (1929), and was in part attributed to variable susceptibility of individual mice to the small amount of free toxin present in the mixture. Although the final testing is carried

out with 20 per cent differences in the amount of toxin in successive mixtures, the value assigned may be closer to the true value than ± 20 per cent, because the results of all the titrations involved are considered in assessing the final titre. It is preferable to carry out several titrations using a small number of mice for each mixture rather than one or two tests with a larger number of mice for each, since technical errors can then better be detected.

In vitro titration.—Flocculation of dysentery neurotoxin and antitoxin has been previously reported (Dumas, Ramon and Bilal, 1926; Halapine, Basilevskaia and Schitkova, 1937; Halapine, 1937; Istrati and Olaru, 1938*a*, *b*; Istrati, 1938*a*, *b*; Boivin and Delaunay, 1940).

Crude toxins (e.g. culture filtrates or autolysates) may contain several antigens, and an antiserum prepared by injection of such a mixture will contain other antibodies in addition to the specific anti-neurotoxin. When mixtures of crude toxin and the corresponding antiserum are made *in vitro*, flocculation may occur in more than one zone due to the interaction of the various components. In order to differentiate the specific neurotoxin-anti-neurotoxin zone from other flocculation zones *two* antisera were selected in the following way:

The potencies of several refined dysentery antitoxic sera were first determined *in vivo* by assay against a test neurotoxin standardized by titration with the British Standard Dysentery Antitoxic Serum. Then mixtures of varying dilutions of these antisera with 1 ml. of a toxin were made, and the positions, with respect to antitoxin unitage, of zones of flocculation were noted. For each serum three zones were present, but only one of each set corresponded with the results of the *in vivo* assays. Calculation of the equivalent of toxin to antitoxin using the other zones led to widely differing values—a tenth, fifteenth or even a thirtieth of that given by the true toxin-antitoxin reaction. Two of the sera with the "non-specific" zones as far apart as possible were chosen as "Laboratory standards," their values in *in vitro* units, corresponding to their specific zones, being regarded as equal to their *in vivo* unitage. For convenience in titrating toxins, dilutions of these sera containing 200, 100 and 50 units/ml. were made in aqueous solution containing 0·9 per cent NaCl and 0·18 per cent of boric acid, the pH being adjusted to 6·5, at which refined anti-neurotoxic sera are most stable.

A preliminary determination of the potency of a toxin was made by blending with a toxin of known potency. Two series of mixtures were prepared containing a constant amount (usually 30 Lf doses) of the known toxin and 1 ml. of the toxin under test. One series was titrated by addition of varying amounts of one of the "Laboratory standard" sera, and the other series titrated similarly with the second serum. When flocculation had occurred the "specific zone" common to the two titrations was used for calculating the titre. A final titration was carried out using the toxin itself with the appropriate range of antitoxin, as indicated by the results of the blend titration. Thus a toxin could be allotted an Lf value, the "flocculating dose" (1 Lf) corresponding to one unit of anti-neurotoxin.

Flocculation took about one hour in mixtures containing equivalent amounts of neurotoxin and antitoxin when maintained in tubes one-third immersed in a water bath at 48–50°. The floccules were small and compact. It was comparatively easy to detect the mixture flocculating first (the equivalent mixture) when the successive amounts of antitoxin varied by 10 per cent.

Toxoids were also titrated by the flocculation method. The time of flocculation with toxoids is usually longer than with toxins.

The ratios of L+ dose to Lf dose were studied for toxins of widely differing potencies. L+ values at the "one unit level" were determined for 26 neurotoxins with potencies

ranging from 5·5 to 33 Lf doses/ml., and at the " five unit level " for 30 neurotoxins with potencies ranging from 18 to 90 Lf doses/ml. The ratio of L+ dose to Lf dose was calculated for each toxin. The ratios for the first group fell between 2·3 and 4·4, and for the second group between 1·6 and 3·0. Grouping the ratios for the " one unit level " in blocks with approximately 20 per cent differences, and the ratios for the " five unit level " in blocks with 10 per cent differences, it was shown that they formed normal distributions about their respective mean values of 3·2 and 2·37 (standard deviations, $\pm$ 0·6 and $\pm$ 0·36 respectively). The high ratios suggest that dysentery toxin contains a considerable amount of toxoid which is capable of flocculating with antitoxin, but which does not, of course, contribute to the toxicity. An alternative explanation is that the most rapidly flocculating complex (the indicator tube in the *in vitro* titration) may contain twice as much antitoxin as is required to " neutralize " the toxin *in vivo*.

The higher ratio obtained when titrating at the " one unit level " is due to the fact that the L+ dose at that level is greater than at the " five unit level," because the amount of uncombined toxin required to provide a toxic mixture is a significantly greater proportion of the comparatively small number of lethal doses of toxin in the mixture at the " one unit level." A contributory cause may be that antitoxin combines more readily with toxin than with toxoid (cf. Glenny, Hopkins and Pope, 1924; Glenny, 1931, for diphtheria toxin). If this preference were greater the more dilute the solution, proportionately more toxin would be required to give a toxic mixture at the " one unit level," where the reagents are more dilute, than at the " five unit level."

Since the ratio of the L+ dose to the Lf dose is reasonably constant, a knowledge of the Lf dose of a toxin enables the determination of the L+ dose to be carried out with greater economy of time and animals, because it is possible to select the appropriate toxin range and start with 50 per cent differences in the amounts of toxin used. Without this it was formerly necessary to start with a wide range of 100 per cent differences and then to narrow the range in subsequent tests.

Production of Dysentery Neurotoxin.

Two general methods have been used for the production of dysentery toxin. The first method entails growth of the organisms in a liquid medium for a considerable time, killing the culture and filtration through a Berkefeld candle. The second method is to grow the bacteria on a solid medium for a short time, remove the growth, suspend it in saline solution and allow autolysis to occur before filtration. Various modifications have been introduced from time to time (Weil, 1943).

The original method of preparation of the toxin used in these laboratories (Okell and Blake, 1930) was to grow a " Smooth " strain of *S. dysenteriae* in an alkaline broth (pH 7·8), containing Chapoteaut peptone, for 12 to 14 days, when the pH rose to 8·6 to 8·8; the organisms were removed by filtration through a Berkefeld candle. Later the method was altered to growth for 24 hours on the surface of a solid agar medium containing papain digest of horse muscle, washing off the growth in saline solution, allowing autolysis to occur at 37° for 5 days in the presence of toluene, followed by filtration through paper pulp and then Berkefeld candles. By this means toxins were obtained having a Minimum Lethal Dose (M.L.D.) for mice of 0·0025 to 0·005 ml., an L+ value (" five unit level ") of 0·2 to 0·6 ml., an Lf value varying between 20 and 45 units/ml., and containing about 2·0 mg. N/ml.

The Lf values for toxins DS 841 B, C and D, DS 846 A_2 and A_5 and DS 856 C, D and E (Table I), obtained by the latter method, show that 85 per cent or more of the toxin is liberated during the first 48 hours of autolysis.

TABLE I.—*Lf Values and Purity of Toxins from Washed and Unwashed Organisms.*

Toxin number.	Duration of autolysis.	Lf/ml.	Mg. N/ml.	Lf/ Mg. N.	Toxin liberated (percentage of total yield).
From Unwashed Organisms.					
DS 841 A	None	11·5	1·55	7·4	20
841 B	24 hours	38	1·63	23·2	85
841 C	4 days	40	1·61	24·8	89
841 D	5 „	45	1·57	28·6	100
842	5 „	34	1·98	17·2	100
843	5 „	28	1·84	15·2	100
846 Bb	None	<5	1·71	<2·9	<9·5
846 A_2	48 hours	53	2·04	26·0	96
846 A_5	5 days	55	2·01	27·4	100
853	None	8	1·57	5·1	14·5
853 A_7	5 days	34	2·15	15·8	62
From Washed Organisms.					
DS 846 Bb	None (washing)	<5	1·71	<2·9	<8·0
846 Ba_2	48 hours	63	0·22	286	100
853	None (washing)	8	1·57	5·1	14·5
853 C	5 days	55	0·32	172	100
855 A	None (washing)	10	1·98	5·0	8·7
855 C	5 days	115	0·65	177	100
855 D	5 „	34	0·18	191	98
856 A	None (washing)	6	2·45	2·5	4·0
856 C	24 hours	125	0·72	174	83
856 D	24–48 „	22	0·18	122	14·5
856 E	2–5 days	4	0·09	44·5	2·7
856 C_1	5 „	32	0·22	145	100

Within quite wide limits the density of the suspension of organisms allowed to autolyse had little effect on the yield of toxin. For instance, toxin DS 855 C was obtained by suspending in 600 ml. of 0·9 per cent NaCl solution the washed organisms from surface cultures in 200 Roux bottles and by allowing autolysis to proceed for 5 days, when the filtrate contained 115 Lf units/ml. At the same time 30 ml. of the original suspension was diluted to 100 ml. and allowed to autolyse for 5 days; the resulting toxin, DS 855 D, had 34 Lf units/ml.; corrected for dilution this value is equivalent to 113 Lf units/ml. Similarly toxins DS 856 C, D and E together contained 151 Lf units/ml. and were obtained from a dense suspension (organisms from 200 Roux bottles in 500 ml.), whilst toxin DS 856 C_1, made by autolysing a 1 in 5 dilution of the same suspension, contained 32 units/ml., corresponding to 160 Lf units/ml. calculated for the original concentration of organisms.

Autolysates from organisms grown on silica gel medium (Anderson and Macsween, 1942) give a toxin having an M.L.D. of 0·025 ml., an Lf value of 26 units/ml. and containing 0·52 mg. N/ml. The yield was about 1 mg. solid/ml.

Purification of Dysentery Neurotoxin.

Since the toxin is a protein and most of the impurities in toxic filtrates are nitrogenous substances derived from the medium and from the breakdown of bacterial bodies, it is obvious that the ratio of the activity of a toxin to its nitrogen content, expressed as the number of Lf units/mg. N, will be a measure of purity. The purer the toxin the higher will be ratio, Lf/mg. N. The efficacy of the various methods of purification to be described has been estimated in this way.

Preliminary purification.

The Lf values of samples DS 841 A and DS 846 Bb (Table I) show that the supernatant fluid obtained by immediate centrifugation of the suspension of organisms collected from the surface of the solid medium in Roux bottles contained little toxin, but about 90 per cent of the nitrogen which would have appeared in the autolysates (*cf.* DS 842 or DS 843). Consequently a considerable purification of autolysate toxins was achieved by twice washing the organisms, by centrifugation and resuspension, prior to autolysis.

The process of washing was greatly facilitated by the use of a "Rough" strain ("Bucharest Rough," kindly supplied by Dr. Boivin), which separated readily from the suspending fluid and yielded as much toxin as "Smooth" strains. The process was also improved by using a De Laval centrifuge to separate the bacterial debris from the autolysate mixture, and filtering the resulting fluid through "Ford SB" sterilizing pads in a Seitz-type filter.

Table I shows that the improvement in purity of the toxin is about tenfold (from an Lf/mg. N ratio of about 20 to about 200), and that such toxins contain about 0·2 to 0·3 mg. N/ml.

As would be expected, prolonged autolysis results in toxins of lower purity, since larger amounts of nitrogenous substances from the bacteria are set free. This is illustrated by toxins DS 856 C, D and E produced by autolysis for 1, 1 to 2 and 2 to 5 days and having Lf/mg. N ratios of 174, 122 and 44·5 respectively.

Precipitation with ammonium sulphate.

Todd (1904) showed that the toxin of Shiga's bacillus could be precipitated from broth culture filtrates by the addition of ammonium sulphate. The method used in the present work was to add measured amounts of solid ammonium sulphate to samples of toxin obtained by autolysis of unwashed organisms in 0·9 per cent NaCl solution at 37°. The resulting precipitated toxin was collected by centrifugation, washed with ammonium sulphate solution of the strength used for precipitation, and dried, first on porous plates in the cold room and then in a vacuum desiccator over conc. sulphuric acid. The ammonium sulphate in the dried product was removed by shaking the finely powdered material with chloroform in a stoppered separating funnel, when the sulphate sank to the bottom and the toxin floated and was readily removed. The toxin was re-treated with chloroform once or twice and finally dried.

The degree of purification obtained by this method was somewhat variable, but, with 30 per cent ammonium sulphate was of the same order as that given by washing the organisms prior to autolysis, although a considerable loss of toxin occurred during the process. Concentrations of ammonium sulphate higher than 30 per cent led to less pure products (Table II).

TABLE II.—*Precipitation of Dysentery Neurotoxins by Ammonium Sulphate.*

The initial toxins were autolysates of unwashed organisms.

Toxin sample.	Precipitated by ammonium sulphate %.	Lf.	L+ dose (5 unit level).	M.L.D. mg.	mg. N/mg.	Lf/mg. N.	Yield. mg./ml.	Recovery of Lf units %.
DS 682 C	0	270 ml.	0·018 ml.	..	..	..	..	..
682 D	30	12 mg.	..	..	0·10	114	1·5	5·6
682 E	50	6·2 mg.	..	..	0·11	57	5·1	9·5
682 F	Satd.	6·7 mg.	..	..	0·125	54	0·18	0·3
699	0	85 ml.	0·17 ml.	..	..	..	..	..
699 C	30	23 mg.	0·5 mg.	0·005	0·11	216	1·32	35·5
757	0	62 ml.	0·18 ml.	..	..	..	..	..
757 A	30	21 mg.	0·57 mg.	0·01	0·069	306	1·18	40·5

Precipitation with trichloroacetic acid.

As was shown by Boivin, the toxin can be precipitated from solution by the addition of 2 per cent of trichloroacetic acid. In the present work it was found necessary to maintain the solutions and reagents used at cold-room temperature (4°) in order to avoid loss of toxin. Chilled 20 per cent trichloroacetic acid solution was added in amount equal to one-tenth of the volume of the chilled toxin solution, the mixture allowed to stand in the cold room for 30–60 mins. and the precipitate separated by centrifugation. The solid was washed twice with cold 2 per cent trichloroacetic acid solution, then three times with cold ethanol and finally twice with cold ether before drying in a vacuum desiccator over conc. sulphuric acid. The toxin was thus obtained as an almost white, friable powder. Less thorough drying by solvents before the final stage gave brown, horny products.

Toxins precipitated by trichloroacetic acid are twice as pure as those obtained by precipitation with ammonium sulphate or from washed organisms, and about 25 times as pure as crude autolysate toxins. The recovery is also very good (Table III). In

TABLE III.—*Precipitation of Dysentery Neurotoxin by Triclhoroacetic Acid.*

Toxin sample.	Precipitation by trichloro-acetic acid %.	Lf/mg.	M.L.D. mg.	mg. N/mg.	Lf/mg. N.	Yield mg./ml.	Recovery of Lf units %.
DS 682 C	2 (from concd. toxin)	32·5	..	0·10	325	7·4	89
699 A	2	65	..	0·11	590	1·25	96
803 A	2	58	0·025	0·11	515	0·83	105
803 A_4	2 (repptd. at pH4)	58	0·05	0·12	505	0·75	100
813 B	2	21	0·025	0·11	206	0·71	83
824 A	1	45	0·025	0·103	438	0·93	124
850 C_1	2	37	..	0·12	300	1·08	153

many cases there was an apparent increase in the amount of toxin, as expressed by Lf units, on precipitation. This might be due to an increased ease of flocculation, and may be correlated with the fact that dialysed toxins give no flocculation reaction, although the toxins obtained from them by precipitation with trichloroacetic acid or cadmium chloride flocculate in a normal manner.

When the mother liquors, remaining after precipitation of the toxin by trichloroacetic acid, were treated with three volumes of ethanol a precipitate of polysaccharide was obtained. This substance had an M.L.D. for mice of about 5 mg. and contained about 1·9 per cent. N. It was obtained from autolysates of both "Rough" and "Smooth" organisms in a yield of about 2 mg./ml. and was apparently not the somatic O-antigen, since it gave no precipitin reaction with anti-O sera. It is possible that this substance was derived from the agar used in the medium and owed its toxicity to a small amount of adsorbed toxic protein. In support of this view are the following facts: The polysaccharide gave a precipitin reaction with an anti-agar serum and probably accounts for one of the "non-specific" zones observed in flocculation. Antisera prepared by injection of the polysaccharide reacted with the protein, whilst anti-protein sera protected mice against the lethal action of the polysaccharide. The yield of polysaccharide from autolysates of washed organisms was much smaller (0·1 to 0·3 mg./ml.) than from unwashed organisms, whilst autolysates from organisms grown on silica-gel medium, containing no agar, yielded no polysaccharide. It should be pointed out, however, that the toxicity of the polysaccharide fraction was not affected by treatment with formaldehyde, which suggests that the toxicity may be a property of the polysaccharide itself and not due to adsorbed protein (neurotoxin) which would be converted into toxoid by formaldehyde.

Precipitation with cadmium chloride.

The neurotoxin could be precipitated from autolysates, either from washed or unwashed organisms, by the addition of a solution of cadmium chloride to give a final concentration of the salt between 1 and 4 per cent. The presence or absence of sodium citrate, or the proportion of sodium citrate to cadmium chloride, appeared to have little effect on the amount of the precipitate or on its quality (cf. the behaviour of diphtheria toxin as reported by Eaton, 1936; Linggood, 1941). The toxin could be recovered from the precipitate by elution with 4 per cent sodium bicarbonate solution at pH 9·5, and separation of the cadmium as the carbonate. Disodium phosphate could be used, but was not so satisfactory as it gave rise to colloidal solutions which made separation of the residue by centrifugation difficult.

Purification of autolysates from unwashed organisms by this means gave rather poor yields (10 to 40 per cent recovery) of toxin having about 200 Lf/mg. N. Treatment of autolysates from washed organisms resulted in recovery of about 80 per cent of the toxin, which contained about 600 Lf/mg. of N (see Table IV). Toxins prepared by this method are the purest that we have yet obtained.

TABLE IV.—*Precipitation of Dysentery Neurotoxins by Cadmium Chloride.*

Autolysates from unwashed (u) or washed (w) organisms were used.

Toxin sample.	Precipitation by $CdCl_2$ %.	Lf/ml.	Lf/mg. N.	Recovery of Lf units %.
DS 842 D	0·5 (u)	11·2	140	33
839 D	0·25 (u)	9·5	176	16·6
839 F	0·5 (u)	5·3	279	9·3
859 A_2a	1·7 (w)	47	645	72
859 B_1a	1·0 (w) + 0·4 per cent Na citrate	57	588	87·5

Precipitation by protamine sulphate.

The toxic protein obtained by precipitation from autolysate toxins by 2 per cent of trichloroacetic acid could be further purified by treatment with protamine sulphate, using 1 mg. of the latter to 2·5 mg. of toxin. The toxin remained in solution whilst the non-specific protein was precipitated. The recovery was of the order of 80 per cent, the toxin containing up to 550 Lf/mg. N. Similar treatment of a toxic protein purified by cadmium chloride and already containing about 550 Lf/mg. N resulted in no further purification and no precipitate was obtained.

Adsorption.

Some purification of the toxic protein was achieved by shaking a solution containing 2 mg. of the protein per ml. of 0·01 N sodium carbonate solution (pH 8·5) with kaolin, which adsorbed the non-specific protein preferentially; an improvement of Lf/mg. N value from 218 to 320 was obtained. Charcoal was found to adsorb the toxin completely, whilst alumina had little effect. Magnesium oxide adsorbed toxin and non-specific protein equally under the conditions of the experiment. Good purification, increasing the purity of the toxin from 218 to 680 Lf/mg. N, was obtained by passing the protein solution through a dry column of magnesium oxide and then "developing" the column with borate buffer at pH 8·7. The recovery, however, was only about 23 per cent, probably because of incomplete elution.

Physical properties.

The toxin, as precipitated by trichloroacetic acid, was a white flocculent solid. After drying *in vacuo*, following washing with ethanol and ether, the toxin was a white to slightly yellow powder. The toxin prepared in the latter way was insoluble in water, in dilute acid and in sodium chloride solutions containing up to 6 per cent. NaCl, but readily soluble in 0·01 N sodium carbonate solution (pH 8·4). The protein was soluble in sodium citrate solution with a maximum solubility, judged by recovery of Lf units, at a concentration of 1 per cent of sodium citrate (pH 7·7). The toxin appeared to be more soluble than the protein present as impurity, since the citrate solution contained 400 Lf/mg. N compared with 240 Lf/mg. N in the original toxin. It was insoluble in formamide, and in dioxan.

The toxin, dissolved in 0·01 N sodium carbonate solution, had a specific rotation—

$$[\alpha]_{D}^{21} - 36° \text{ and } [\alpha]_{5461}^{21} -42° \quad (c = 1{\cdot}0).$$

Precise information about the size of the toxin molecule is not available, but it is small enough to pass through a Berkefeld candle or Seitz EK filter-pad. The toxin is retained by "Cellophane" "sausage skin" during dialysis, and by membranes prepared from 5 per cent collodion solution supported on filter-paper or on Berkefeld candles. Solutions of the toxin (autolysate filtrates) can be concentrated by ultrafiltration through such membranes.

After dialysis the toxin lost its power of flocculating with antiserum; the activity could be restored by the addition of sufficient sodium carbonate to make the solution 0·01 N. Addition of sodium hydroxide or of sodium phosphate to give a pH value equal to that attained in the presence of 0·01 N sodium carbonate did not restore the flocculating power, nor did the addition of sodium chloride up to 0·9 per cent. The lethal dose of the toxin for mice was not altered by dialysis.

The stability of the toxin to heat at various pH values was determined by adjusting the pH of samples of a solution containing 1 mg. of toxin per ml. of 0·01 N sodium bicarbonate to values between pH 5·5 and 9·4, subjecting the samples to heat at various temperatures for 30 minutes, readjusting the pH values to 7·5 and determining the Lf titre. The toxin was found to be most stable at pH 7·0 to 7·5, losing only half its flocculating power at 100° in half an hour. In more acid and more alkaline solutions the loss was greater, the flocculating power falling to one-third in half an hour at 100° at pH 5·5, and being completely lost in the same time at 90° at pH 9·4. The toxin was not coagulated by heat.

Chemical properties.

The toxin had the properties of a protein, giving the usual reactions in the biuret, Millon and xanthoproteic tests. It contained some carbohydrate which was not removed by extraction with water, dioxan or formamide, nor by repeated precipitation with trichloroacetic acid or cadmium chloride. Extraction with 90 per cent phenol removed the polysaccharide but denatured the protein at the same time.

The protein contained:

Total N	10·6 per cent.	
Amino N	0.58 ,,	("copper method" of Pope and Stevens, 1939).
	(Values corrected for ash.)	
Total P	0·15 per cent.	
Inorganic P	0·03 ,,	
	(All values corrected for moisture.)	

TABLE V.—*Changes in Toxicity and Flocculation Associated with the Conversion of Dysentery Neurotoxin into Toxoid.*

Toxin sample.	Origin of toxin.	Values before treatment.			Treatment with—	Duration of toxoiding. weeks.	Values after treatment.		
		M.L.D. for mice.	Lf doses.	L+ at 1 unit level. ml.			M.L.D. for mice.	Lf doses/ml.	L+ at 1 unit level.
DS 633	Autolysate of unwashed organisms	0·00125 ml.	30/ml.	0·1	0·3% formaldehyde	5	0·1 ml.	30	0·25 ml.
871 E	Autolysate of washed organisms	0·0025 ml.	27/ml.	..	0·3% „	7	0·5 ml.	21	Non-toxic
815 B	Purified by trichloro-acetic acid	0·0125 mg.	21/mg.	0·08	0·3% „	2	>2·5 mg.	0	„
813 B	Ditto	0·0125 mg.	21/mg.	0·08	0·01% „	2	0·25 mg.	16	„
813 B	„	0·0125 mg.	21/mg.	0·08	0·5% hexamethylene-tetramine	7	0·125 mg.	13	0·18 mg.
894 E	Purified by $CdCl_2$	0·0025 ml.	58/ml.	..	0·3% formaldehyde	1	>0·5 ml.	0	Non-toxic
868Bc	„	0·005 ml.	27/ml.	0·08	0·05% „	9	>0·5 ml.	13	„

Titration curves of the toxin using trichloroacetic acid or hydrochloric acid showed that the protein had an iso-electric point at about pH 6. Precipitation began at pH 5·3 and was complete at pH 4. The protein combined somewhat slowly with acid as shown by the change of pH with time after addition of acid to a solution of the protein.

The dysentery neurotoxin protein was attacked by trypsin, pepsin and activated papain, with liberation of amino-nitrogen and change in the optical rotation. The flocculating power and the toxicity to mice were affected as follows:

	Loss of flocculating power %	Loss of toxicity %
Trypsin	100	100
Pepsin	60–85	60–85
0·04 N HCl at 45°	25–40	little
Activated papain, pH 8·4	little	,,
,, ,, pH 7·0	15–40	15–40

Conversion into toxoid.

The toxin in autolysates from unwashed organisms was not completely converted into toxoid by 0·3 per cent formaldehyde at pH 8·2 and 37°, even after contact for several months, but the flocculation value remained high. The action of 0·3 per cent formaldehyde on purified toxins (obtained by precipitation with trichloroacetic acid or cadmium chloride) led to rapid and complete loss of both toxicity and flocculating power. Toxins from washed organisms occupied an intermediate position, being rendered almost completely non-toxic, but retaining moderate flocculating power. The probable explanation is that with crude toxins most of the formaldehyde is taken up by non-specific nitrogen and so prevented from acting on the toxin, whereas with purified toxin this cannot occur and the toxin is destroyed by excess of formaldehyde. Purified toxins were successfully treated by the use of 0·01 per cent formaldehyde, with almost complete loss of toxicity, but with retention of high flocculating power. Similar results were obtained by the use of 0·5 per cent of hexamethylenetetramine at pH 7·0, since formaldehyde is liberated slowly and in small quantities. These findings are illustrated by the data in Table V.

The Use of Dysentery Toxoid for Immunization.

Toxoids prepared by treatment of autolysate toxins from washed organisms with 0·1 per cent formaldehyde are in regular use for the production of dysentery antitoxic sera in horses; the average antitoxin titre of such sera, prepared by the methods in use in these laboratories, has been 3300 units/ml., with occasional values up to 10,000 units/ml.

Preliminary trials of active immunization against dysentery toxin have been made in human volunteers, and support the findings of Farrell, Fraser and Ferguson(1944) that useful increases of antitoxin content are induced and that the reactions are slight. Agglutinins for Shiga's bacillus could not be detected in the sera after immunization. The method seems worthy of further trial, and in view of the results of Morgan and Schütze (1943) with the somatic O-antigen, it is suggested that an antigen containing the latter as well as toxoid should be a particularly effective prophylactic immunizing agent.

SUMMARY.

1. An *in vivo* method of titrating the neurotoxin of *Shigella dysenteriae* against dysentery antitoxic sera (giving L+ values) with the use of small groups of mice is described. The toxin can also be titrated *in vitro* by flocculation with antitoxin to give Lf values, the selection of the specific zone of flocculation being facilitated by blending with a toxin of known value.

2. The ratio, L+ dose/Lf dose, is approximately constant for all the toxin samples investigated. This ratio is high, suggesting that the toxins contain an appreciable amount of material which is non-toxic, but capable of flocculating with antitoxin.

3. The autolysis of washed *S. dysenteriae* organisms of a "Rough" strain yields toxins containing about 200 Lf doses/mg. of N. These toxins were further purified by precipitation with trichloroacetic acid or with cadmium chloride to give products containing 500 and 600 Lf doses/mg. of N respectively.

4. The purified toxins had the properties of a protein, and were destroyed by trypsin and partially destroyed by other proteolytic enzymes.

5. Purified dysentery toxins were converted into toxoids by 0·01 per cent formaldehyde at pH 8, or by 0·5 per cent hexamethylenetetramine at pH 7, with but little loss of flocculating value.

REFERENCES.

ANDERSON, C. G., AND MACSWEEN, J. C.—(1942) *J. Path. Bact.*, **54**, 530.
BLAKE, A. V., AND OKELL, C. C.—(1929) *Ibid.*, **32**, 120.
BOIVIN, A., AND DELAUNEY, A.—(1940) *C. R. Soc. Biol.*, **133**, 376.
Idem AND MESROBEANU, L.—(1937*a*) *Ibid.*, **124**, 442.—(1937*b*) *Ibid.*, **125**, 796.—(1937*c*) *Ibid.*, **126**, 222.—(1937*d*) *Ibid.*, **126**, 232.—(1937*e*) *Ibid.*, **126**, 652.—(1937*f*) *C. R. Acad. Sci. Paris*, **204**, 302.—(1938) *C. R. Soc. Biol.*, **128**, 446.
CLAUBERG, K. W., AND SARTORIUS, F.—(1942) *Dtsch. Militarärzt*, **7**, 310. Quoted in *Bull. War Med.*, **3** (1942), 215.
DUMAS, J., RAMON, G., AND BILAL, S.—(1926) *Ann. Inst. Pasteur*, **40**, 134.
EATON, M. D.—(1936) *J. Bact.*, **31**, 347, 367.
FARRELL, L., AND FERGUSON, H.—(1943) *Canad. J. publ. Hlth.*, **34**, 130.
FARRELL, L., FRASER, D. T., AND FERGUSON, H.—(1944) *Ibid.*, **35**, 311.
FORSYTH, W. L. (1942) *Indian J. med. Res.*, **30**, 213. Quoted in *Bull. Hyg.*, **17** (1942), 800.
GLENNY, A. T.—(1931) *System of Bacteriology*, Med. Res. Coun., Chap. IV, 140.
Idem, HOPKINS, B. E., AND POPE, C. G.—(1924) *J. Path. Bact.*, **27**, 261.
HALAPINE, K.—(1937) *Ann. Inst. Pasteur*, **58**, 599.
Idem, BASILEVSKAIA, L., AND SCHITKOVA, N.—(1937) *Ibid.*, **58**, 154.
ISTRATI, G.—(1938*a*) *C. R. Soc. Biol.*, **129**, 1013.—(1938*b*) *Z. ImmunForsch.*, **94**, 264.
Idem AND OLARU, A.—(1938*a*) *C. R. Soc. Biol.*, **127**, 1338.—(1938*b*) *Ibid.*, **129**, 399.
KESTERMANN, E., AND VOGT, K. E.—(1941) *Klin. Wschr.*, **20**, 739.
LINGGOOD, F. V.—(1941) *Brit. J. exp. Path.*, **22**, 255.
MORGAN, W. T. J., AND SCHÜTZE, H.—(1943) *Lancet*, ii, 284.
OKELL, C. C., AND BLAKE, A. V.—(1930) *J. Path, Bact.*, **33**, 157.
POPE, C. G., AND STEVENS, M. F.—(1939) *Biochem. J.*, **33**, 1070.
PRIGGE, R.—(1937) *Zbl. Bakt.*, I (Orig.), **140**, 230.—(1940) *Klin. Wschr.*, **19**, 337.
SHIGA, K.—(1898) *Zbl. Bakt.*, **24**, 817, 870, 913.—(1903) *Ibid.*, **29**, 327.
TODD, C.—(1904) *J. Hyg., Camb.*, **4**, 480.
WEIL, A. J.—(1943) *J. Immunol.*, **46**, 13.

THE INFLUENCE OF COAGULASE ON THE PHAGOCYTOSIS OF STAPHYLOCOCCI.

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THE association of potential pathogenicity of staphylococci with power to produce coagulase suggests that this substance may play a part in the initiation and development of staphylococcal infections. In a previous paper we showed that coagulase requires activation before it can convert fibrinogen into fibrin clot, and that this activator substance is present in the plasma of some species and absent from that of others (Smith and Hale, 1944). It is perhaps significant that fowls, rats and mice, which are devoid of activator substance, are also particularly resistant to experimental infection with coagulase positive staphylococci. Preliminary experiments convinced us that this resistance is largely due to the rapidity and efficiency with which injected cocci are engulfed and removed by phagocytes, and led to the logical deduction that inhibition of phagocytosis by coagulase activity might be a determining factor in the infection of susceptible species. This paper reports the *in-vitro* experiments designed to test the validity of this hypothesis.

MATERIALS AND METHODS.

Citrated and defibrinated blood.

Blood was obtained from volunteers by venepuncture and from animals by cardiopuncture. Half the sample was prevented from clotting by the addition of sodium citrate solution. The other half was defibrinated by shaking with glass beads, citrate solution being added subsequently to equalize the sodium citrate concentrations of both specimens. These operations were performed in an incubator room at 37° C.

Washed blood suspensions.

A measured quantity of citrated blood was centrifuged and the plasma discarded. The cellular deposit, after washing once with physiological saline at 37° C., was resuspended in saline to the original volume.

Leucocyte suspensions.

Exudative leucocytes were obtained from rabbits, guinea-pigs and rats by the method elaborated by Hamburger (1926). Briefly this consists in filling the peritoneal cavity with 0·9 per cent. saline, and, after 3–4 hours, withdrawing the opalescent fluid through a large bore needle drilled with lateral holes. The fluid is received into a mixture of equal parts of 0·7 per cent saline and 1·1 per cent sodium citrate solution, allowing three parts of the peritoneal fluid to one of the mixture. When centrifuged this fluid yields a cellular deposit which, after washing once in physiological saline, can be resuspended in saline to give a leucocyte suspension of any required density. In our hands this technique resulted in suspensions consisting of 95 per cent polymorphonuclear leucocytes. The animals suffered no injurious effects, and after a rest of 14–28 days could be used again.

Organisms.

All organisms used in these experiments were added as measured quantities of 18-hour digest broth cultures.

Phagocytosis.

Apart from a few early experiments made by Wright's capillary pipette method (Wright and Douglas, 1903), a rotating tube technique was employed throughout. Ingredients were measured into small, rubber-stoppered, pyrex glass tubes, which were then rotated at 8 r.p.m. on a revolving drum. At the end of rotation films were made, stained by Leishman's stain, and counts of the organisms contained in at least 100 polymorphonuclear leucocytes made independently by two or more observers. Counts as recorded in the tables represent the number of organisms per 100 polymorphonuclear leucocytes. The complete experiment unless otherwise stated was performed in an incubator room at 37° C.

THE INFLUENCE OF THE PRESENCE OF FIBRINOGEN UPON PHAGOCYTOSIS OF COAGULASE POSITIVE STAPHYLOCOCCI.

Phagocytosis in citrated and defibrinated blood.

The phagocytosis of non-coagulase producing organisms was approximately the same in citrated and defibrinated blood. With the exception of one experiment the phagocytosis count was always slightly higher in defibrinated blood; this is probably due to the different physical properties possessed by the two fluids. Coagulase positive staphylococci always showed a marked difference in the phagocytosis counts, defibrinated blood being at least twice as effective as citrated blood. This phenomena was exhibited by three strains of coagulase positive staphylococci using blood from three different volunteers. It was also shown by rabbit blood.

TABLE I.—*Phagocytosis of Coagulase Positive and Coagulase Negative Organisms in Citrated and Defibrinated Blood.*

Organism.	Coagulase.	Blood donor.	Organisms per 100 polymorphs.		D/C.
			Defibrinated blood (D).	Citrated blood (C).	
Staph. A1	—	A	281	336	0·8
,,	—	B	387	350	1·1
,,	—	C	133	97	1·4
,,	—	D	367	293	1·3
Subtilis.	—	C	406	289	1·4
Staph. albus	—	A	559	518	1·1
Staph. E.	+	A	2239	1091	2·1
,,	+	Rabbit	718	158	4·5
Staph. III	+	B	2172	856	2·5
Staph. IV	+	D	463	161	2·9
,,	+	A	2242	888	2·5

The ratios D/C shown in Table I for the coagulase negative organisms are seen to be near unity and relatively constant over the range of organisms tested. This is experimental evidence that the opsonic contents of citrated and defibrinated blood are approximately equal. The remarkable D/C ratios of the coagulase producers must therefore be due to inhibition of phagocytosis brought about by the organism itself in the presence of citrated blood. The chief difference between citrated and defibrinated

blood is the presence of fibrinogen in the former and its absence in the latter, so that the reduced phagocytosis in citrated blood most probably depends upon an interaction between fibrinogen and coagulase.

The time factor in resistance to phagocytosis.

Although phagocytosis of coagulase positive staphylococci is reduced in citrated blood it is not completely inhibited. It therefore seemed desirable to elucidate more fully the nature of this reduction. Estimation of the phagocytosis rates for coagulase and non-coagulase producers were made over a period of 20 minutes. In all experiments the coagulase negative staphylococci were phagocytosed at a steadily progressive rate both in citrated and defibrinated blood. Initially the same is true of the coagulase positive staphylococci, but after a period of time the phagocytosis in citrated blood

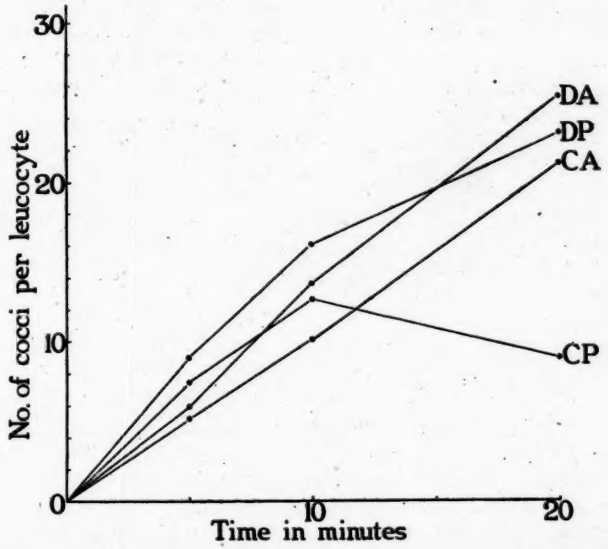

FIG. 1.—The phagocytosis of coagulase positive and coagulase negative staphylococci in citrated and defibrinated blood. D. Defibrinated blood. C. Citrated blood. A. Coagulase negative *Staph. albus*. P. Coagulase positive Staph. IV.

virtually ceases. The results of a typical experiment are illustrated in Fig. 1. All coagulase positive staphylococci tested gave a similar result, the variable factor for each strain being the period of time elasping before phagocytosis ceased. This time interval appeared to be proportional to the organism's coagulase production capacity. An energetic coagulase producer allowed phagocytosis in citrated blood for a very short period, whereas Fig. 1 demonstrated phagocytosis of the feebler coagulase producer Staph. IV taking place over a period of 10 minutes. Apparently phagocytosis proceeds until such time as the inhibitory mechanism is mobilized. It is suggested that the inhibitory mechanism is the interposition of a fibrin barrier at the surface of the organism due to the action of coagulase upon fibrinogen.

The agglutination of organisms and leucocytes.

The stained films exhibited agglutination of organisms of approximately equal degree both in citrated and defibrinated blood. The agglutination of coagulase positive staphylococci by citrated blood is, however, of a very different character from that produced by defibrinated blood. In the latter type of agglutination, shown also by non-coagulase producing organisms irrespective of the nature of the medium, individual cell outlines are still clearly visible. In the former type the cell outlines of the cocci in the agglutinated masses are obscured and the masses, surrounded by platelets, stain purplish black with Weigert's fibrin stain, indicating that fibrin is deposited either around or in close proximity to the cocci.

When phagocytosis of coagulase positive staphylococci in citrated blood has proceeded for a certain length of time, not only are the cocci agglutinated, but also a large number of the polymorphonuclear leucocytes are found in tight compact bundles. Examination of these aggregates reveals a large number of cocci, a few of them ingested, but the great majority free in the midst of the leucocytes. The picture suggests that the leucocytes, attracted towards agglutinated cocci, are able to ingest only a few of them before becoming entangled and immobilized by fibrin. We consider that this agglutination and immobilization of leucocytes is probably also a factor in the initiation of a staphylococcal infection.

The fall in the curve, Staph. IV citrated blood, in Fig. 1 is, to some extent, due to this agglutination of leucocytes. So long as the clumps consist of only a few leucocytes, samples withdrawn for filming are representative of the whole mixture, but this is no longer the case later on when large aggregates have formed. As phagocytosis has virtually ceased at this stage, films devoid of the large masses fail to reveal the phagocytosis which has occurred earlier.

PHAGOCYTOSIS OF STAPHYLOCOCCI PREVIOUSLY CONDITIONED BY COAGULASE ACTIVITY.

In the previous experiments organisms, phagocytes and the opsonin containing fluid were all brought into immediate contact with each other. Such a state of affairs could occur but rarely *in vivo*. An organism having gained access to the tissues, the probable sequence of events is transudation of the tissue fluids followed by progressive invasion of phagocytes. Further experiments were therefore designed in order to study the effect on phagocytosis of preliminary treatment of the cocci with plasma. Cultures were rotated for 10 minutes at 37° C. with plasma or serum before adding the phagocytic medium. Table II demonstrates clearly the remarkable inhibition of phagocytosis of coagulase positive staphylococci receiving preliminary treatment with a coagulable plasma. This inhibition was a constant finding independent of the proportions existing between culture, plasma and phagocytic medium. The initial treatment with plasma allows the organism to mobilize its coagulase defence mechanism before the phagocytes enter the field.

With reagents from species having non-coagulable plasmas there were no significant differences between the plasma and serum counts excepting only in the case of Staph. III with guinea-pig reagents. We were unable to reconcile this anomalous result with our working hypothesis until we re-investigated the coagulase properties of Staph. III. It was then found to be capable of clotting both human and guinea-pig plasma at 37° C. Similar differences between the species ranges of coagulase activity amongst different strains of staphylococci were reported by Walston (1935). The

results in Table III show that its unusual power of coagulation does not embrace mouse or rat plasma, both of which had proved to be entirely devoid of the inhibitory effects of guinea-pig plasma. Thus Staph. III provided unexpected confirmation of the hypothesis that the defence mechanism of coagulase positive staphylococci is dependent upon their opportunity to interact with susceptible fibrinogen.

TABLE II.—*The Effect of Previous Treatment with "Coagulable" or "Non-Coagulable" Plasma upon the Resistance of Coagulase Positive Staphylococci to Phagocytosis.*

	Organism.	Donor of phagocytes plasma and serum.	Time of phagocytosis in mins.	Cocci per 100 polymorphs. Serum (S).	Plasma (P).	S/P.
"Coagulable" plasma.	*Staph. E	Rabbit (leucocytes)	20	529	90	5·88
	* ,,	,, ,,	10	120	16	7·5
	,,	,, ,,	40	390	106	3·68
	,,	,, ,,	20	188	42	4·48
	Staph. IV	Human (defib. blood)	5	510	177	2·88
	,,	,, (cit. blood)	5	230	93	2·47
"Non-coagulable" plasma.	*Staph. E	G. pig (leucocytes)	20	503	402	1·25
	* ,,	,, ,,	10	106	88	1·20
	Staph. IV	,, (cit. blood)	10	815	902	0·90
	,,	,, (defib. blood)	10	653	553	1·18
	,,	,, (washed blood)	10	1554	1334	1·16
	Staph. III	Mouse (,,)	10	90	88	1·02
	,,	Rat (leucocytes)	5	332	218	1·52
	,,	G. pig (,,)	10	485	43	11·28
	,,	,, (cit. blood)	10	1439	281	5·12
	,,	,, (defib. blood)	10	1035	195	5·31

* Experiment performed at room temperature.

TABLE III.—*The Coagulase Properties of Staph. III and Staph. E.*

Organism.	Citrated plasma diluted 1 in 10. Human.	Guinea-pig.	Mouse.	Rat.
Staph. III	25	78	—	—
Staph. E	25	—	—	—

The figures represent the times of clot formation in minutes.

There remained the possibility that inhibition of phagocytosis in the presence of fibrinogen might be due less to the formation of a protective barrier around the cocci than to the removal of leucocytes from the field of action by their aggregation, or merely to an increased viscosity of the menstruum. Such effects may indeed play a part, but the two experiments summarized in Table IV show clearly that coagulase activity is chiefly directed to a specific protection of the coagulase producing organisms. Equal quantities of a mixed culture of a coagulase producing and a non-coagulase producing organism were added to tubes containing human plasma and human serum. After preliminary rotation for 10 minutes, leucocytes were added to each tube and films made after a further period of rotation. Inhibition of phagocytosis in the plasma tube was limited to the staphylococci; the second non-coagulase producing organism present remained just as vulnerable as when treated with serum devoid of fibrinogen.

TABLE IV.—*The Phagocytosis of Mixed Cultures Previously Treated with Human Plasma or Serum.*

Mixed culture.	Organisms per 100 polymorphs.			
	Serum.		Plasma.	
	Staph. III.	2nd organism.	Staph. III.	2nd organism.
Staph. III + diphtheroid	1341	350	336	495
Staph. III + *Cl. Welchii*	1439	68	324	71

INHIBITION OF PHAGOCYTOSIS IN "NON-COAGULABLE" PLASMA PLUS COAGULASE ACTIVATOR.

The contention that opportunity for the exercise of coagulase activity largely determines the resistance of staphylococci to phagocytosis receives additional support from the results of the four experiments given in Table V. In each experiment reagents were obtained from a species having a plasma non-coagulable by the particular strain of staphylococcus used. The organisms and phagocytes were rotated in mixtures containing serum or plasma, both with and without the addition of coagulase activator in the form of a little fresh human serum. The ineffectiveness of the organism's coagulase defence mechanism in a non-coagulable plasma is confirmed, but the conversion of the plasma into a coagulable substrate by activator substance allows this mechanism full play. The low count in the activated plasma mixture of the experiment with mouse reagents is especially significant because the sample of human serum used as activator on this occasion had obviously a very high content of opsonins, evidenced by the counts in the two serum mixtures.

TABLE V.—*Inhibition of Phagocytosis of Coagulase Positive Staphylococci in a Medium Containing a "Non-Coagulable" Plasma plus Activator.*

Organism.	Donor of phagocytes, plasma and serum.	Cocci per 100 polymorphs.			
		Serum.	Plasma.	Serum + Activator.	Plasma + Activator.
Staph. IV	G. pig (washed blood)	1150	1120	1676	367
,,	,, ,, ,,	1554	1334	1401	33
Staph. III	Mouse (washed blood)	90	88	292	36
,,	Rat ,, ,,	218	332	255	44

DISCUSSION.

The experiments reported prove beyond reasonable doubt that coagulase activity specifically inhibits the phagocytosis of coagulase producing organisms in appropriate *in-vitro* mixtures. Conditions *in vivo* are much more complex and the aggressive armoury of the staphylococcus undoubtedly includes many other factors, such as alpha toxin and leucocidins, in addition to coagulase. It is a natural assumption however that, during the process of an infection, the coagulase confers upon the organism a resistance against the host's first line of defence, thus enabling it to elaborate its toxic products. It is significant that species susceptibility to infection is correlated with plasma coagulability. Non-susceptible species can tolerate relatively enormous doses of coagulase positive staphylococci, suggesting that in the absence of a coagulable menstruum the bacteria are extremely vulnerable to the primary clearing mechanism of the host.

The exact mechanism of this inhibition of phagocytosis remains obscure. Wright and Douglas (1903, 1904) showed that effective phagocytosis depends upon preliminary sensitization of bacteria by opsonins, and subsequently several workers have demonstrated that the opsonization is related to an alteration of the surface physical properties of the organisms. (Broom and Brown, 1930; Lucké, Mudd, S., Mudd, E. B. H., McCutcheon and Strumia, 1927, 1929, 1930, 1933.) It is conceivable that the formation of a fibrin envelope around the cocci either prevents opsonization or nullifies the surface changes which opsonization has produced. In experiments not reported in this paper we have demonstrated that the monocytes are just as subject to this inhibitory mechanism as are the polymorphonuclear leucocytes.

The formation of fibrin may be of assistance to the invading staphylococci in yet another way. Birch-Hirschfeld (1934) reported that coagulase positive staphylococci are agglutinated by human plasma. Agglutination of non-coagulase producers by the normal opsonins may also occur, but Berger (1943) pointed out that this is of a different nature and suggested that with coagulase positive organisms the phenomenon is due to local precipitation of fibrinogen round the cocci. Our observations support this view and we believe that the resultant compact masses, embedded in a fibrin matrix, form a mechanical obstacle to phagocytosis quite apart from the specific mechanism of inhibition. Leucocytes which approach the coccal aggregates become entangled in the fibrin and themselves clump together so that large masses are formed, consisting of free cocci and leucocytes both with and without ingested bacteria. These may well confront the macrophages which subsequently enter the zone of infection, with an insoluble problem and thus help to determine the localized nature of the lesion characterized by abundant pus formation.

In our previous paper we showed that non-coagulable plasmas of certain species could be rendered coagulable by the addition of activator substance in the form of fresh human or rabbit serum, or testicular extract. It now appears that plasma, activated in this manner, promotes inhibition of phagocytosis of the staphylococci just as readily as does a normally coagulable plasma. Can plasma be thus activated *in vivo*? If so it is possible that an artificial susceptibility to infection could be conferred upon non-susceptible species. Investigations on these lines are now in progress.

SUMMARY.

1. Phagocytosis of coagulase positive staphylococci is inhibited in the presence of coagulable plasma. The initial protection afforded to the organism is specific and is not dependent upon a physical alteration of the medium, for the phagocytosis of other species of bacteria present at the same time is unaffected.

2. Inhibition of phagocytosis is demonstrable with the plasmas of certain species not normally coagulable, provided that a little coagulase activator is added to the mixture.

3. The agglutination of both organisms and leucocytes which results from coagulase activity confers a further protection upon the staphylococci.

4. The possible significance of these phenomena in the initiation and development of staphylococcal lesions is discussed.

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REFERENCES.

BERGER, F. M.—(1943) *J. Path. Bact.*, **55**, 435.

BIRCH-HIRSCHFELD, L.—(1934) *Klin. Wschr.*, **13**, 331.

BROOM, J. C., AND BROWN, H. C.—(1930) *Brit. J. exp. Path.*, **11**, 305.

HAMBURGER, H. J.—(1926) *In. Abderhalden E., Handb. d. biolog. Arbeitsmethoden*, Abt. IV, Teil 4, Heft 3, 953.

LUCKÉ, B., STRUMIA, M., MUDD, S., MCCUTCHEON, M., AND MUDD, E. B. H.—(1933) *J. Immunol.*, **24**, 455.

MUDD, S., AND MUDD, E. B. H.—(1927) *J. exp. Med.*, **46**, 173.

MUDD, S., LUCKÉ, B., MCCUTCHEON, M., AND STRUMIA, M.—(1929) *Ibid.*, **49**, 779.

MUDD, S., LUCKÉ, B., AND STRUMIA, M.—(1933) *J. Immunol.*, **24**, 493.

SMITH, W., AND HALE, J. H.—(1944) *Brit. J. exp. Path.*, **25**, 101.

STRUMIA, M., MUDD, S., MUDD, E. B. H., LUCKÉ, B., AND MCCUTCHEON, M.—(1930) *J. exp. Med.*, **52**, 299.

WALSTON, H. D.—(1935) *J. Hyg. Camb.*, **35**, 549.

WRIGHT, A. E., AND DOUGLAS, S. R.—(1903) *Proc. Roy. Soc.*, B, **72**, 362.—(1904) *Ibid.*, **73**, 128.